PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12P 19/34, C12N 15/00, C07H 21/00, 21/02, 21/04

A1

(11) International Publication Number: **WO 00/09738**

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18808

(22) International Filing Date: 17 August 1999 (17.08.99)

(30) Priority Data:

60/096,830	17 August 1998 (17.08.98)	US
60/102,535	30 September 1998 (30.09.98)	US
60/106,910	3 November 1998 (03.11.98)	US
60/106,885	3 November 1998 (03.11.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/096,830 (CON)
Filed on	17 August 1998 (17.08.98)
US	60/102,535 (CON)
Filed on	9 September 1998 (09.09.98)
US	60/106,910 (CON)
Filed on	3 November 1998 (03.11.98)
US	60/106,885 (CON)
Filed on	3 November 1998 (03.11.98)

(71) Applicant *(for all designated States except US)*: PACKARD BIOSCIENCE COMPANY [US/US]; 800 Research Parkway, Meriden, CT 06450 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: WOODWARD, Karen, L. [US/US]; 3 Woodward Road, Columbia, CT 06237 (US). NALLUR, Girish, N. [IN/US]; 599D Prospect Street, New Haven, CT 06511 (US). TAYLOR, Seth [US/US]; Apartment 2–9B, 100 Memorial Drive, Cambridge, MA 02142 (US).

(74) Agent: MYERS, P., Louis; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ROLLING CIRCLE–BASED ANALYSIS OF POLYNUCLEOTIDE SEQUENCE

(57) Abstract

Disclosed are methods of detecting a polynucleotide sequence.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

ROLLING CIRCLE-BASED ANALYSIS OF POLYNUCLEOTIDE SEQUENCE

Cross Reference to Related Applications

The application claims benefit from: provisional application Serial No. 60/096,830, filed August 17, 1998; provisional application Serial No. 60/102,535, filed September 9, 1998; provisional application Serial No. 60/106,910, filed November 3, 1998 and; provisional application Serial No. 60/106,885, filed November 3, 1998.

Background of the Invention

The invention relates to a method of analyzing a polynucleotide sequence.

Background of the Invention

Current genomics and diagnostics procedures can rely upon accurate and simultaneous detection and quantitation of a large number of nucleic acids from complex biological sources. Because biological materials are often present in limiting amounts, it is often necessary to amplify one or more nucleic acid fragments from the sample before subjecting them to quantitation and/or detection methods.

Methods for nucleic acid amplification described include PCR, NASBA, 3SR, SDA. However, where quantitation of one or more nucleic acids in complex mixtures of nucleic acids is desired, these methods can provide less than optimal results. Spurious amplification products may arise due to mispriming or mismatched hybridizations of primer oligonucleotides. Further, multiplexing of amplification reactions can sometimes result in one or more products being underrepresented in the final products.

Summary of the Invention

The invention features methods for sensitively detecting the presence of a nucleic acid in a sample. Described herein are methods for synergistic multiplexed amplifications of nucleic acids. Multiple individual chemical and biochemical reactions for target identification, amplification, cleavage to unit lengths, and partitioning and detection of each signal independently of other similar signals in the multiplexed reaction can be caused to occur simultaneously in a single tube or device as part of an isothermal process.

In one aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally,

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and

(c) cleaving the product to produce cleaved amplified product,

wherein the oligonucleotide multimer is more sensitive to cleavage than is the circular template, thereby analyzing a polynucleotide.

In preferred embodiments the method further includes:

(3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positionally distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region; hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments the oligonucleotide multimer is cleaved and the circular template is not cleaved.

In preferred embodiments the circular template has one or more nucleotide or modified nucleotide which is resistant to cleavage. While the modified nucleotide is resistant to cleavage, when it serves as a template, it nevertheless incorporation of the appropriate complementing nucleotide.

In preferred embodiments the circular template, but preferably not the oligonucleotide multimer, includes one or more of deoxy uracil, or a methylated or hemimethylated base.

In preferred embodiments cleavage of the oligonucleotide multimer is effected by hybridization of a cleavage probe (thus forming a ds cleavage site) and said cleavage probe is chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence

complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. (Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.)

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic

fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated

reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is

a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality),

b) has a variable region capable of hybridizing adjacent to the genetic event; and

has a 3' end capable of serving as a priming site for extension;

hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and

using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In another aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally, (ii) at least one nucleotide effective to produce a cleavage site;

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide product complementary to the circular oligonucleotide template; and

(c) cleaving the oligonucleotide product at the cleavage site to produce the cleaved amplified product,

wherein the formation of said oligonucleotide product and its cleavage occur simultaneously, thereby analyzing a polynucleotide.

In preferred embodiments the method further includes: (3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positionally distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region;

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. (Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used in methods described herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array,

(preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in U.S. Patent No. 5,714,320. Generally, the definitions provided for circular vectors and their amplification in U.S. Patent No. 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls.

In another aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally, (ii) optionally at least one nucleotide effective to produce a cleavage site;

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide product complementary to the circular oligonucleotide template, and optionally

(c) cleaving the oligonucleotide product at the cleavage site to produce the cleaved amplified product,

wherein at least one component, e.g., the TRCA primer, the sample, or the product is immobilized.

In preferred embodiments the method further includes: (3) analyzing said product from (2) b or c.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments one or more of the immobilized components is immobilized such that they are positionally distinguishable.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference, can be used in methods described herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array,

(preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in U.S. Patent No. 5,714,320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In another aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a plurality of single-stranded circular templates, wherein each single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally, (ii) optionally at least one nucleotide effective to produce a cleavage site, and (iii) an identifier sequence which allows it to be distinguished from at least one other circular template of the plurality;

(b) combining the circular templates with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide product complementary to a circular oligonucleotide template, and optionally

(c) cleaving the oligonucleotide product at the cleavage site to produce the cleaved amplified product, thereby analyzing the sample sequence.

In preferred embodiments the method includes: (3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positionally distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region complementing to an identifier sequence,

hybridizing the product with the array of capture probes.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S

restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template

for an enzyme mediated reactions. For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used in methods described herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array,

(preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In another aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally, (ii) at least one nucleotide effective to produce a cleavage site;

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template, wherein the oligonucleotide multimer comprises multiple copies (amplified) of the sample sequence; and optionally

(c) providing an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product;

and (3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon cleavage at the cleavage site.

In other preferred embodiments, the signal decreases upon cleavage at the cleavage site.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S

restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions. For example, after hybridization to the

capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. ,631,134, both of which are hereby incorporated by reference can be used, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event

hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In another aspect, the invention features a method of analyzing a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence;

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template, wherein the oligonucleotide multimer comprises multiple copies (amplified) of the sample sequence; and optionally

(c) providing an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety;

and (3) analyzing said product from (2) b or c, thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon annealing of the first and second detection oligonucleotides.

In other preferred embodiments, the signal decreases upon annealing of the first and second detection.

In preferred embodiments, the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. (Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another primer, preferably a TRCA primer, in this or other methods disclosed herein.).

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions. For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used, particularly the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes

providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the

region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positionally distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5,714,320, and all other U.S. patents mentioned herein are incorporated by reference.

In another aspect, the invention features, a rolling circle vector. The vector includes:

a first nucleic acid contacting region which is complementary to a first portion of a target nucleic acid;

a binding site for a type IIS restriction enzyme, wherein the cleavage site for the type IIS restriction enzyme is located within the first nucleic acid contacting region;

optionally,a primer target sequence, which can be used to provide for amplification of the vector when it circularized;

a second type IIS restriction enzyme binding site;

a second nucleic acid contacting region that is complimentary to a second portion of the target nucleic acid and which includes the cleavage site for the second type IIS restriction enzyme, wherein the first and second target portions are disposed such that upon hybridization of the vector to the target the ends of the vector can be joined, e.g., by ligation.

In preferred embodiments the elements are in the recited order.

In preferred embodiments the first and second type 2s restriction enzymes sites are recognized by the same type IIS restriction enzyme. In other preferred embodiments the first type IIS restriction enzyme site is for a first type IIs restriction enzyme and the second type IIS restriction enzymes site is for a second type IIS restriction enzyme.

In another aspect, the invention features, a method of providing a nucleotide fragment containing a target sequence. The method includes:

providing a target nucleic acid sequence;

contacting the target nucleic acid sequence with a vector having,

a first nucleic acid contacting region which is complementary to a first portion of a target nucleic acid;

a binding site for a type IIS restriction enzyme, wherein the cleavage site for the type IIS restriction enzyme is located within the first nucleic acid contacting region;

optionally,a primer target sequence, which can be used to provide for amplification of the vector when it circularized;

a second type IIS restriction enzyme binding site;

a second nucleic acid contacting region that is complimentary to a second portion of the target nucleic acid and which includes the cleavage site for the second type IIS restriction enzyme, wherein the first and second target portions are disposed such that upon dybridization of the vector to the target the ends of the vector can be joined, e.g., by ligation;

allowing the first nucleic acid contacting region and the second nucleic acid contacting region to hybridize to said target;

joining the end of the first nucleic acid contacting region with an end of said second nucleic acid contacting region, for example by ligating the ends together;

optionally, allowing rolling circle amplification to proceed to produce a molecule having a plurality of copies of the vector;

contacting the vector or amplified vector with type IIs restriction enzyme sufficient to generate a nucleotide fragment containing a target sequence.

In another aspect, the invention features, an excision vector. The vector includes:

a first nucleic acid contacting region which is complimentary to a first portion of a target nucleic acid (in preferred embodiments one or more nucleotides of this region can vary in a population of vectors, for example one vector could have (t) at an interrogation position while another vector could have (a) at the interrogation position);

optionally a primer sequence which allows for rolling circle amplification of the vector;

optionally a spacer region;

a sequence tag region, which preferably include four to ten nucleotide, which can allow for identification of the vector;

a cleavage site for example a site for cleavage by a restriction enzyme;

a second nucleic acid contacting region which in complimentary to a second portion of the target nucleic acid, wherein the first and second target portions are disposed such that upon hybridization of the vector to the target the ends of the vector can be joined, e.e., by ligation.

In another aspect, the invention features, a plurality of the above vectors, wherein the plurality include the vector having a first nucleotide at the interrogation position and a vector having a second nucleotide at the interrogation position.

In preferred embodiments the plurality prefers includes vectors having each of A,C,T,G, at the interrogation site.

In another aspect, the invention features, a method of analyzing a target sequence. The method includes:

providing a target sequence;

contacting the target sequence with a vector having

a first nucleic acid contacting region which is complimentary to a first portion of a target nucleic acid (in preferred embodiments one or more nucleotides of this region can vary in a population of vectors, for example one vector could have (t) at an interrogation position while another vector could have (a) at the interrogation position);

optionally a primer sequence which allows for rolling circle amplification of the vector;

optionally a spacer region;

a sequence tag region, which preferably include four to ten nucleotide, which can allow for identification of the vector;

a cleavage site for example a site for cleavage by a restriction enzyme;

a second nucleic acid contacting region which in complimentary to a second portion of the target nucleic acid, wherein the first and second target portions are disposed such that upon hybridization of the vector to the target the ends of the vector can be joined, e.e., by ligation.

optionally allowing rolling circle amplification to proceed. Preferred embodiments of the method include hybridizing cleavage products of the rolling circle amplified vector to an identifier probe for example, a probe, located on an ordered array of probes, thereby allowing analysis of the sample, for example by identification of the identity of the interrogation nucleotide.

In another aspect, the invention features, a pair of oglionucleotides useful for forming a cleavage site on an amplified rolling circle vector product. The set of oglionucletides includes:

a 5' oglionucletide having a restriction enzyme binding site and a spacer region which is three prime to said binding site;

a 3' oglionucletide which contains a sequence tag for example a sequence of five to eight nucleotide which allows for identification of a given oglionuclotide and a mixture of other oglionuclotides;

wherein said 5' and 3' oglionuclotides hybridize to adjacent region on a rolling circle amplification product, and where upon cleavage of a double stranded duplex formed by hybridization of the 5' and 3' oglinucleotide to the linear product, a fragment is produced which contains the oglinuclotides sequence tag at its three primed end.

In another aspect, the invention features, method of analyzing a target nucleic acid sequence. The method includes:

providing a target sequence;

contacting a rolling circle vector with said target and allowing rolling circle amplification to proceed;

contacting the amplified rolling circle product with a 5' oglionucletide having a restriction enzyme binding site and a spacer region which is three prime to said binding site;

a 3' oglionucletide which contains a sequence tag for example a sequence of five to eight nucleotide which allows for identification of a given oglionuclotide and a mixture of other oglionuclotides; wherein said 5' and 3' oglionuclotides hybridize to adjacent region on a rolling circle amplification product, and where upon cleavage of a double stranded duplex formed by hybridization of the 5' and 3' oglinucleotide to the linear product, a fragment is produced which contains the oglinuclotides sequence tag at its three primed end,

thereby analyzing the target nucleotide.

In preferred embodiments the fragments which contain a nucleotide sequence on the 3' prime end are analyzed by contacting with a identifier probe for example an ordered array of identifier probes.

In another aspect, the invention features, a set of oglinucleotides for analyzing a target sequence. The set include a first and a second oglinuclotide wherein; the first oglinuclotide includes preferably in the recited order, regions A,B,C, (optionally D), E, B'1, and F, wherein region A and region F can hybridize to regions A' and F' respectively on the target sequence and wherein A' and F' are positioned so that such hybridization of A and F to A' and F' allows hybridization between B and B' of the first oglionuclotide, and wherein the duplex formed by B and B' forms a cleavage site which upon cleavage results in the formation of linear cleavage product which includes region C, optionally D, and region E;

The second oglionucleotide includes region C' and region E prime, wherein region C' is complimentary to region C and region E', is complimentary to

region E on the first vector and wherein contact of the second oglionuclotide product with the cleavage product and hybridization of C to C' and E to E' result in circularizing the C optionally D, E, cleavage product.

In another aspect, the invention features, a method of analyzing a test nucleotide in a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2) (a) annealing an effective amount of sample polynucleotide sequence to a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally,

(b) combining the circular template with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and , optionally,

(c) cleaving the product to produce cleaved amplified product.

In preferred embodiments, the sample polynucleotide sequence is provided by:

(i) hybridizing a first primer to a nucleic acid sample template. The first primer has a DNA sequence which is sufficiently complementary to a RNA sequence of the

first nucleic acid sample template;

(ii) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first nucleic acid sample template. The first DNA sequence and the first primer comprise a second template;

(iii) separating the first template from the second template to allow hybridization of a second primer;

(iv) hybridizing the second primer to the second template. The second primer has a DNA sequence which is sufficiently complementary to a DNA sequence of the second template. The second primer also has an antisense sequence of a promoter and an antisense sequence of a transcription initiation site for a RNA polymerase;

(v) synthesizing a second DNA sequence covalently attached to the second primer and complementary to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer. The second and third DNA sequences, the second primer and the second template comprise a third template; and

(vi) synthesizing a plurality of copies of the RNA sequence of the first template from the third template to provide the sample polynucleotide sequence. Methods disclosed in U.S. Patent Nos. 5, 130,238 and 5,409,818, hereby incorporated by reference, can be used in this and other methods described herein.

In preferred embodiments, the products of rolling circle amplification can be further amplified by:

(i) synthesizing an RNA sequence of the reaction products from (2) b or c to generate a first nucleic acid sample template;

(ii) hybridizing a first primer to a region of the RNA sequence of step (i). The first primer has a DNA sequence which is sufficiently complementary to a RNA sequence of the first nucleic acid sample template;

(iii) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first nucleic acid sample template. The first DNA sequence and the first primer comprise a second template;

(iv) separating the first template from the second template to allow hybridization of a second primer;

(v) hybridizing the second primer to the second template. The second primer has a DNA sequence which is sufficiently complementary to a DNA sequence of the second template. The second primer also has an antisense sequence of a promoter and an antisense sequence of a transcription initiation site for a RNA polymerase;

(vi) synthesizing a second DNA sequence covalently attached to the second

primer and complementary to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer. The second and third DNA sequences, the second primer and the second template comprise a third template; and

(vii) synthesizing a plurality of copies of the RNA sequence of the first template from the third template to provide additional copies of the products of the rolling circle amplification. Methods disclosed in U.S. Patent Nos. 5, 130,238 and 5,409,818, hereby incorporated by reference, can be used in this and other methods described herein.

In preferred embodiments, the test nucleotide, or its complementary nucleotide, upon cleavage is sufficiently close to an end of the cleaved amplified

product such that its presence can be detected by its effect on a reaction which involves the end nucleotide of the cleavage product.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments, the sample nucleic acid serves as a primer. In other preferred embodiments synthesis is primed with a nucleic acid other than the sample nucleic acid.

In preferred embodiments, the reaction is a hybridization reaction. In other preferred embodiments, the reaction is a ligation reaction, a polymerization reaction, e.g., a DNA polymerase catalyzed reaction, modification, or a restriction or other cleavage reaction.

In preferred embodiments, the test nucleotide, or its complementary nucleotide, is within 1 (i.e., it is at the end), 2, 3, 4 or 5 base pairs from the end of the cleaved amplified product.

Numerous variations in the circular oligonucleotide template are within the invention. As is described herein, the circular oligonucleotide template can be formed, e.g., in the reaction mix, from a linear precursor. As is described herein, the circular oligonucleotide can be resistant to cleavage, providing for more efficient amplification. Other variants are described below.

In preferred embodiments, the circular oligonucleic template is prepared by a process which includes the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a positioning oligonucleotide, e.g., a sample sequence, (wherein the positioning oligonucleotide has a 5' nucleotide sequence complementary to a portion of the

sequence which includes the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence which includes the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned such that they can be joined, e.g., as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. (Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another in this or other methods disclosed herein.)

In preferred embodiments, the oligonucleotide multimer is more sensitive to cleavage than is the circular template. In preferred embodiments the oligonucleotide multimer is cleaved and the circular template is not cleaved. For example, in preferred embodiments the circular template has one or more nucleotide or modified nucleotide which is resistant to cleavage. While the modified nucleotide is resistant to cleavage, when it serves as a template, it nevertheless incorporation of the appropriate complementing nucleotide. The circular template, but preferably not the oligonucleotide multimer, can include one or more of deoxy uracil, or a methylated or hemimethylated base to render the circular template more resistant to cleavage.

Preferential cleavage of the oligonucleotide multimer can be effected by hybridization of a cleavage probe (thus forming a double stranded cleavage site) and said cleavage probe is

chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of

DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence, cleaves in the region which binds the sample sequence, or cleaves at the target nucleotide.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes differentially to a sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing at least one nucleotide of the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

The products of rolling circle amplification can be analyzed in various ways. In some embodiments, the products of rolling circle application are analyzed on positional arrays.

Accordingly, in preferred embodiments the method further includes:

(3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region;

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes

providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and

using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positional distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positional distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In preferred embodiments the primer, the sample, or the product is immobilized.

In preferred embodiments, the method further includes providing an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product;

and (3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon cleavage at the cleavage site.

In other preferred embodiments, the signal decreases upon cleavage at the cleavage site.

In preferred embodiments, the method further includes providing an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety.

In another aspect, the invention features, a method of analyzing a test nucleotide in a polynucleotide, e.g., detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2) (a) annealing an effective amount of sample polynucleotide sequence to a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally,

(b) combining the circular template with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and

(c) cleaving the product to produce cleaved amplified product,

wherein upon cleavage, the test nucleotide, or its complementary nucleotide, is sufficiently close to an end of the cleaved amplified product such that its presence can be detected by its effect on a reaction which involves the end nucleotide of the cleavage product.

In preferred embodiments, the sample nucleic acid serves as a primer. In other preferred embodiments synthesis is primed with a nucleic acid other than the sample nucleic acid.

In preferred embodiments, the reaction is a hybridization reaction. In other preferred embodiments, the reaction is a ligation reaction, a polymerization reaction, e.g., a DNA polymerase catalyzed reaction, modification, or a restriction or other cleavage reaction.

In preferred embodiments, the test nucleotide, or its complementary nucleotide, is within 1 (i.e., it is at the end), 2, 3, 4 or 5 base pairs from the end of the cleaved amplified product.

Numerous variations in the circular oligonucleotide template are within the invention. As is described herein, the circular oligonucleotide template can be formed, e.g., in the reaction mix, from a linear precursor. As is described herein, the circular oligonucleotide can be resistant to cleavage, providing for more efficient amplification. Other variants are described below.

In preferred embodiments, the circular oligonucleic template is prepared by a process which includes the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a positioning oligonucleotide, e.g., a sample sequence, (wherein the positioning oligonucleotide has a 5' nucleotide sequence complementary to a portion of the sequence which includes the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence which includes the 5' end of the linear precursor oligonucleotide, to yield an open

oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned such that they can be joined, e.g., as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. (Rolling circle amplification can be primed by the positioning oligo nucleotide, e.g., the target nucleic acid, or by another in this or other methods disclosed herein.)

In preferred embodiments, the oligonucleotide multimer is more sensitive to cleavage than is the circular template. In preferred embodiments the oligonucleotide multimer is cleaved and the circular template is not cleaved. For example, in preferred embodiments the circular template has one or more nucleotide or modified nucleotide which is resistant to cleavage. While the modified nucleotide is resistant to cleavage, when it serves as a template, it nevertheless incorporation of the appropriate complementing nucleotide. The circular template, but preferably not the oligonucleotide multimer, can include one or more of deoxy uracil, or a methylated or hemimethylated base to render the circular template more resistant to cleavage.

Preferential cleavage of the oligonucleotide multimer can be effected by hybridization of a cleavage probe (thus forming a double stranded cleavage site) and said cleavage probe is chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence, cleaves in the region which binds the sample sequence, or cleaves at the target nucleotide.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes differentially to a sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing at least one nucleotide of the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

The products of rolling circle amplification can be analyzed in various ways. In some embodiments, the products of rolling circle application are analyzed on positional arrays.

Accordingly, in preferred embodiments the method further includes:

(3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region;

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. 5,503,980 and or U.S. 5,631,134, both of which are hereby incorporated by reference can be used herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality),

b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positional distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positional distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

In preferred embodiments the primer, the sample, or the product is immobilized.

In preferred embodiments, the method further includes providing an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection

moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product;

and (3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon cleavage at the cleavage site.

In other preferred embodiments, the signal decreases upon cleavage at the cleavage site.

In preferred embodiments, the method further includes providing an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety.

In another aspect the invention features a method of analyzing a polynucleotide, e.g., detecting a specific nucleotide sequence or a genetic event in a sample. The method includes:

(1) providing a sample, which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2)(a) annealing an effective amount of sample polynucleotide sequence to an oligonucleotide, wherein the oligonucleotide includes, preferably in a 5' to 3' orientation, a unique region which is complementary to a target sequence in the sample polynucleotide sequence, optionally a spacer region, a first pairing region, a connecting region which preferably is not homologous to the sample sequence, optionally a second spacer region, a second pairing region which is complementary to the first pairing region, and a second unique region

complementary to a second adjacent sequence in the target polynucleotide; wherein hybridization of the unique regions to the sample promotes formation of a duplex between said first and second pairing regions, and wherein said duplex includes a cleavage site;

(b) cleaving the duplex to produce a cleaved product; and

(c) allowing the ends of the cleaved product to be joined, thereby forming a circular molecule,

wherein formation of the circular molecule is indicative of the presence of a sample sequence. Rolling circle amplification can be primed by a primer, preferably a TRCA primer, in this or other methods disclosed herein.

In a preferred embodiment the invention further includes ligating the cleaved product to form a circle and using the circle to promote rolling circle amplification, to produce an oligonucleotide product.

In another preferred embodiment, the method further includes providing a ligation template oligonucleotide which hybridizes to the cleaved product and thereby brings the ends of the cleaved product in proximity for a reaction, e.g., ligation.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of a sequence, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with oligonucleotide.

In preferred embodiments the oligonucleotide includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence or cleaves in the region which binds the sample sequence.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of a sequence, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In a preferred embodiment a region of the oligonucleotide is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes effectually to sample nucleic acid having the event and does not hybridize to a sample nucleic acid not having the event.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In preferred embodiments the oligonucleotide is a single stranded capture probe in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the oligonucleotide product which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions. For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an oligonucleotide product, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The oligonucleotide product which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized oligonucleotide product. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. ,631,134, both of which are hereby incorporated by reference can be used, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a

variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on a three-dimensional array, e.g., a gel array. In preferred embodiments the method includes providing a plurality of single-stranded oligonucleotides, wherein each of the single-stranded oligonucleotdies is positionally distinguishable from other oligonucleotide of the plurality on the array, and wherein each positionally distinguishable oligonucleotide includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

Preferably, an oligonucleotide has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide itself may be constructed of DNA or RNA or analogs thereof. Preferably, the oligonucleotide is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the oligonucleotide and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in 5, 714, 320. Generally, the definitions provided for circular vectors and their amplification in 5, 714,320, apply to terms used herein, unless there is a conflict between the terms in which

case the meaning provided herein controls. 5, 714, 320, and all other U.S. patents mentioned herein are incorporated by reference.

The objectives of the presently described compositions and methods include:

(1) An isothermal process for the quantitative detection of the presence of one or more nucleic acids in a sample by selective amplification of said nucleic acids using a series of chemical and enzymatic steps in a multiplex assay format

(2) Describing assay conditions for integration of enzymatic reactions of the process for target recognition, amplification, and quantitative detection into a single step compatible with automation and high throughput analyses for industrial applications

(3) Describing assay conditions for increasing the overall yield of amplified nucleic acid products by alleviating potential constraints on the amplification targets by simultaneous cleavage and immobilization of amplified products

(4) Describing miniaturized devices for simultaneous analysis of multiple nucleic acid fragments compatible with simplification and cost reduction of analysis and detection instruments

(5) Increasing the fidelity and overall efficiency of the process by spatial segregation of amplification targets or products by selective capture on to separate but distinct elements contained in the device to eliminate competition during the amplification process

(6) Encoding amplification targets with specific nucleic acid sequence tags to detect the quantitative presence of each allele of a nucleic acid or gene present in the mixture of nucleic acids

(7) Describing assay conditions for sequence-specific partitioning of nucleic acid sequence tags by specific hybridization and/or ligation of the nucleic acid tags onto a device containing arrayed oligonucleotides

(8) Describing a variety of detection methods for determining the quantitative presence of nucleic acids in the original mixture. Detection methods include, but are not limited to, fluorescent labels, redox labels and electronic detection methods either by themselves or in conjunction.

Cost efficient and rapid process for quantitative analysis of nucleic acids in a multiplex format

(9) To determine the sequence of target nucleic acids.

The invention provides methods and devices for the quantitative detection of nucleic acids present in a sample. The methods are based on the specific recognition of a target nucleic acid present in the sample by hybridization of the target nucleic acid with an oligonucleotide designed to yield a circular nucleic acid molecule by the action of a nucleic acid ligase, such as DNA or RNA ligase. The covalently closed circle can be amplified by an integrated series of enzymatic and/or chemical reactions to yield multiple copies of linear single stranded replicas of the original circle. Further enzymatic steps can include amplification of circles by polymerases capable of strand displacement and simultaneous preferential cleavage of the linear relative to the parent circular nucleic acid molecule, using, e.g, sequence-specific endonucleases.

Cleaved nucleic acid replicas can be partitioned by specific hybridization and immobilized by sequence specific-recognition onto an array element containing oligonucleotide(s) packaged into the device. Each array element can specifically capture cleaved fragments arising from a single initial recognition event representing the target nucleic acid in the original sample. The signal intensity detected at each element can then reflect the relative abundance of the target molecule relative to other nucleic acid targets or alleles in the original sample. Thus, a multitude of array elements can capture a multitude of cleaved fragment species.

Herein are described methods for the synergistic action of distinct enzyme activities and other biochemical reactions described herein in a

single, integrated, isothermal process for the quantitative detection of nucleic acids. Several alternative strategies are provided for protecting the parent closed circles from cleavage, while at the same time permitting efficient cleavage of the amplification products. While a cleavage step is optional, including this step can increase the overall yield of amplified products.

Also provided herein are assay conditions for multiplexing nucleic acid amplification and detection processes. This allows for the simultaneous analysis of a large number of nucleic acid fragments present in the sample. In the final detection step, a specific quantitative signal is detected for each allele or nucleic acid fragment present in the sample. Spatial separation of the amplification targets into distinct array elements eliminates competition of simultaneously amplifying targets and improves the fidelity and yield of the overall process. Further, the spatial separation provides a direct means of marking amplification targets that yield insufficient or no signals due to failed amplification reactions that potentially arise from failure of priming events or mispriming. These steps minimize generation of ambiguous data and facilitates troubleshooting attempts.

Also described are a variety of detection methods for qualitative and quantitative determination of each nucleic acid or allele present in the sample. Detection methods can rely on , e.g., fluorescent signals, redox pairs, electronic detection, or enzymatic reactions.

Devices for miniaturization and parallel analysis of nucleic acids in a sample can include, e.g., microplates, acrylamide gel pads, flow-through chips, and or other supports.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other

references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figs. 1A-E are a drawings showing the steps and reagents involved in analyzing a target nucleic acid sequence.

Fig. 2 is a schematic drawing of a TRCA vector.

Fig. 3 is a schematic drawing of a drop-out TRCA vector.

Fig. 4 is a schematic drawing of a TRCA excision vector.

Fig. 5 is a schematic drawing of a TRACE vector.

Fig. 6 is a schematic drawing of a TRCA primer.

Fig. 7 is a schematic drawing of a cleavage oligonucleotide.

Fig. 8 is a schematic drawing of a cleavage oligonucleotide formed from two short oligonucleotides.

Detailed Description

Rolling Circle Amplification

Embodiments of the invention are based on the use of circular vectors (as described in 5,714,320) to analyze a sequence, e.g., to sequence the nucleic acid in question, or to identify SNPs, mutations and RNA molecules, or to clarify a sample, e.g., as to disease state, or generally in expression profiling or analysis. The circular vectors can be closed circular

vectors, open circular vectors which when brought into contact with the analyte, have abutting ends which can be covalently linked, e.g., ligated.

Rolling circle amplification (RCA) is used to generate many copies of an oligo preferably with defined ends (as described in 5,714,320). The single-stranded product of rolling circle amplification can be rendered double-stranded by the annealing of un-circularized, complementary probe vector. The ds DNA RCA product can be fragmented, e.g., using a typeIIS restriction enzyme, such that the DNA is cleaved in the middle, or at the ends, of the region generated by the ligation reaction. The dsDNA fragments generated by the restriction digest can be analyzed, e.g., on an array, e.g., an array of indexing linkers (see, e.g., 5,508,169). If the probe vector is labeled with capture or anchoring moiety, e.g., a biotin group, then it is possible to render the dsDNA fragments generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture or anchoring moiety reactive, e.g., strepavidin-labeled, substrates, e.g., magnetic beads or a solid support. The single-stranded DNA fragments can be analyzed on a Cantor array. These oligonucleotides are analyzed on a Cantor Capture array (see, e.g., 5,503,980) using, e.g., fluorescent detection methodology.

In other embodiments, the captured DNA fragments are analyzed using mass spectrometry. Alternatively, the target DNA is applied to a multiplicity of wells and a population of RCA vectors is added to each well. The RCA products are analyzed using mass spectrometry following fragmentation, where the amplification of specific RCA vectors is determined by differences in molecular weight of the RCA product fragments. Multiple RCA vectors can be analyzed simultaneously in a single reaction using this approach.

The steps involved in analyzing a polynucleotide sequence as described herein are depicted in Fig. 1. One or both strands of one or more nucleic acids contained in a sample of nucleic acids, 110 is contacted with a

mixture of nucleic acids containing one or more TRCA vectors, 112, and other reagents. The nucleic acids in the sample constitute single-stranded, double-stranded or partially double stranded DNA or RNA, a mixture of DNA or RNA, DNA-RNA hybrids where one strand is a molecule of DNA and the other strand is a molecule of RNA that may be partially or wholly hybridized to each other with or without gaps. The methods are equally adaptable to the use of composite DNA-RNA hybrids where part of one strand of a molecule is composed of DNA and the remaining part of the same strand is composed of RNA. The nucleic acid or acids in the sample may originate from genomic DNA, total or fractionated RNA from cells and tissues of living organisms or microorganisms, viruses, or recombinant DNA molecules originating from these samples.

Where the contacted nucleic acid is double-stranded, it may first be converted to a single strand of nucleic acid, 111, by one or more methods including, alkali denaturation, heat denaturation, enzymatic treatments, or other methods known in the art. The reagents preferably include enzymes, oligonucleotides and primers, nucleotides, ribo- or deoxyribonucleotides, dideoxyribonucleotides, or other modified nucleotides, detection tags such as fluorophores or other detection moieties tagged to oligonucleotides or in solution, and one or more optional reagents.

In a preferred embodiment, 112 is appropriately hybridized to 111 without gaps and is ligated to create a circular thermophilic rolling circle amplification (TRCA) vector, 113. In the next step, the TRCA primer 114 appropriately hybridized to 113 is extended by the action of a polymerase, preferably a thermostable polymerase exhibiting strand displacement activity, and nucleotide triphosphates to yield a concatamer of single-stranded DNA 115, where each unit of the concatamer is a replica of, and complementary to the sequence and polarity of 112.

This process described in Fig. 1 may optionally include a cleavage step. Typically a cleavage primer, 116 anneals by hybridization to

complementary sites on 115 and reconstitutes a partial double stranded nucleic acid structure 117. The double stranded region preferably contains one or more target sites for a recognition means 118, such as an endonuclease. The recognition means is preferably a restriction endonuclease of the type IIs type, less preferably any restriction endonuclease, or any protein that recognizes and binds to double stranded DNA, preferably resulting in cleavage of the recognized nucleic acid.

In preferred embodiments, cleavage of concatamers 115 results in the formation of a partial duplex such as 117, wherein one strand 119, constitutes a unit length nucleic acid containing the sequence of 111, and the other strand or strands comprises of short oligonucleotides such as, 120 and 121, containing sequences from the cleavage primer, 116. The thermal stability of 120 and 121 is less than that of 116. 120 and 121 therefore dissociate from 119 at elevated temperatures. In preferred embodiments, the Tm of 120 and 121 is considerably less than the operating temperature of the process.

In preferred embodiments, the cleavage oligonucleotide 116 contains a fluorophore 123 and quencher 124 pair. In this implementation, cleavage of 122 after hybridization to 119 and digestion by a restriction endonuclease releases the quenching effect of 124 and is detected as an increase in fluorescence intensity of 123. The methods are equally adaptable to alternate placements of 123 and 124 at any position along the length of the oligonucleotide 116.

In alternative embodiments, the cleavage oligonucleotide 116 is composed of 2 separate oligomers 125 and 126, that together constitute the sequence of 116. In preferred embodiments, 125 and 126 hybridize without gaps to adjacent sets of nucleotides contained in 119 and are stabilized by base stacking. In such an implementation, moieties 123 and 124 preferably constitute a FRET pair in a homogeneous assay. Preferably, one or more

nucleotides in either 125 or 126 or both contain degenerate positions, modified nucleotides or additional modifications.

In alternative implementations of the process described in Fig. 1, an optional reporter oligonucleotide 127 is present. 127 is composed, at least in part, of ribonucleotides, preferably a composite DNA:RNA hybrid oligonucleotide. 128, the RNA portion of 127, is partly or wholly complementary to any region of 119 or its concatamer and is typically 50 to 75 nucleotides in length, more preferably 30-60 nucleotides in length, and most preferably, 10-25 nucleotides in length. In alternative embodiments, 127 is composed of two distinct oligomers 131 and 132, which together constitute the sequence of 127. In preferred embodiments, 131 and 132 hybridize without gaps to adjacent sets of nucleotides contained in 119 and are stabilized by base stacking.

The DNA segments, 129 and 130 are each preferably 10-20 nucleotides in length, more preferably 7-15 nucleotides in length, and most preferably 5-8 nucleotides in length. The sequence of nucleotides contained in 129 and 130 are preferably complementary only in part, or not at all, to any region of contiguous bases in any of the nucleic acids or oligonucleotides contained in the reaction mixture. 129 and 130 preferably contain reporter moieties 133 and 134, that constitute any of, but not limited to, detection tags such as fluorophores that form either a FRET-quench or FRET pair, more preferably a FRET pair, or chemiluminescent tags, haptens, capture or capturing moieties, amplification tags, or other modifications know in the art that provide a selective advantage or enhance the overall efficiency of the process. Either 129 or 130, or both, may optionally be partially or wholly double stranded. Optionally, sequences for additional recognition means or amplification may be encoded in 129 or 130, or both.

When reporter oligonucleotides contain ribonucleotides, detection signals can be generated using a ribonuclease protection assay.

Positional Arrays

Positional arrays suitable for the present invention include high and low density arrays on a two dimensional or three dimensional surface. Positional arrays include nucleic acid molecules, peptide nucleic acids or high affinity binding molecules of known sequence attached to predefined locations on a surface. Arrays of this nature are described in numerous patents which are incorporated herein by reference, Cantor US 5,503,980; Southern EP 0373 203 B1; Southern 5,700,637 and Deugau 5,508,169. The density of the array can range from a low density format, e.g., a microtiter plate, e.g., a 96- or 384- well microtiter plate, to a high density format, e.g. 1000 molecules/cm2, as described in Fodor US 5,445,934.

The surface on which the arrays are formed can be two dimensional, e.g., glass, plastic, polystyrene, or three dimensional, e.g. polymer gel pads, e.g. polyacrylamide gel pads of a selected depth, width and height.

In preferred embodiments, the target or probes bind to (and can be eluted from) the array at a single temperature. This can be effected by manipulating the length or concentration of the array or nucleic acid which hybridizes to it, by manipulating ionic strength or by providing modified bases.

Proximity Methods

Proximity methods include those methods whereby a signal is generated when a first member and second member of a proximity detection pair (e.g., a first and second detection moiety) are brought into close proximity.

A "proximity detection pair" will have two members, the first member, e.g., an energy absorbing donor or a photosensitive molecule and the second member, e.g., an energy absorbing acceptor or a chemiluminescer

particle. When the first and second members of the proximity detection pair are brought into close proximity, a signal is generated.

Examples of proximity methods include the following:

Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is based on a donor fluorophore that absorbs a photon of energy and enters an excited state. The donor fluorophore transfers its energy to an acceptor fluorophore when the two fluorophores are in close proximity by a process of non-radiative energy transfer. The acceptor fluorophore enters an excited state and eliminates the energy via radiative or non-radiative processes. Transfer of energy from the donor fluorophore to acceptor fluorophore only occurs if the two fluorophores are in close proximity.

Homogeneous time resolved fluorescence (HTRF)

Homogeneous time resolved fluorescence (HTRF) uses FRET between two fluorophores and measures the fluorescent signals from a homogenous assay in which all components of the assay are present during measurement. The fluorescent signal from HTRF is measured after a time delay, thereby eliminating interfering signals. One example of the donor and acceptor fluorophores in HTRF include europium cryptate [(Eu)K] and XL665, respectively.

Luminescent oxygen channeling assay (LOCI)

In the luminescent oxygen channeling assay (LOCI), the proximity detection pairs includes a first member which is a sensitizer particle that contains phthalocyanine. The phthalocyanine absorbs energy at 680nm and produces singlet oxygen. The second member is a chemiluminescer particle that contains olefin which reacts with the singlet oxygen to produce chemiluminescence which decays in one second and is measured at 570nm. The reaction with the singlet oxygen and the subsequent emission depends on the proximity of the first and second members of the proximity detection pair.

Gel Pad Arrays

Gel pad arrays can be used as ordered arrays in methods described herein. Gel pads, including arrays of gel pads, can be prepared by a variety of methods, some of which are known in the art. For example, see, e.g., Timofeev et al., Nucleic Acids Research (1996), Vol. 24, 3142-3148; Drobyshev et al., Gene (1997) 188: 45-52; Livshits et al., Biophysical Journal (1996) 71:2795-2801; Yershov et al., Proc. Natl. Acad. Sci. USA (1996) 93:4913-4918; Dubiley et al., Nucleic Acids Research (1997), Vol. 25, 2259-2265; and U.S. Patent No. 5,552,270 by Khrapko et al.; each of the foregoing is incorporated herein by reference. Gel pad arrays are the preferred positional arrays for use in the methods described herein.

On some embodiments, a sample which contains a target (analyte) polynucleotide, such as a sample which contains genomic DNA, is loaded into a gel pad. An array of gel pads on a first solid support can be employed to perform an analysis on a plurality of samples, and or using a plurality of probes to detect a plurality of characteristics, e.g., SNPs, of a sample or samples. The genomic DNA is preferably digested (e.g., with a restriction enzyme) to provide shorter fragments of DNA which can easily diffuse into the gel pad(s). The gel pad composition and/or the size of fragments can be selected to permit the target polynucleotides to diffuse into the gel pad, and/or to prevent larger pieces of, e.g., genomic DNA from diffusing into the gel pad. The small volume of the gel pad(s) (preferably less than about 1 microliter, more preferably less than about 500, 100, 50, 10, 5, 1, 055, or 0.1 nanoliters per gel pad), permits the diffusion of reactants and target to occur in a conveniently short time period (e.g., preferably less than 5, 2, 1, 0.5, or 0.1 minutes). After the sample polynucleotide has diffused into the gel pad, the remaining sample can be washed away.

An "array" can be any pattern of spaced-apart gel pads disposed on a substrate; arrays can be conveniently provided in a grid pattern, but other

patterns can also be used. In preferred embodiments, a gel pad array includes at least about 10 gel pads, more preferably at least about 50, 100, 500, 1000, 5000, or 10000 gel pads. The array is an array of gel pads of substantially equal size, thickness, density, and the like, e.g., to ensure that each gel pad behaves consistently when contacted with a test mixture. In certain embodiments, however, the pads of a gel pad array can differ from one another; e.g., a mixed gel pad array can be constructed which includes more than one size or type of gel pad, e.g., gel pads made of different gel materials, or which entrap different species such as reagents or polynucleotide probes. In certain preferred embodiments, gel pads in an array are less than about 1 mm in diameter (or along a side, e.g., in the case of square gel pads), more preferably less than about 500 microns, still more preferably less than about 100, 75, 50, 25, 10, 5, or 1 micron in diameter.

A gel pad can have any convenient dimension for use in a particular assay. In preferred embodiments, a gel pad is thin enough, and porous enough, to permit rapid diffusion of at least certain reaction components into the gel pad when a solution or suspension is place din contact with the gel pad. For example, in one embodiment, a gel pad array for use in sequencing by hybridization permits polynucleotide fragments from a sample mixture to diffuse (within a conveniently short time period) into the gel pads and hybridize to oligonucleotide capture sequences disposed within the gel pads. In certain preferred embodiments, a gel pad (e.g., in an array of gel pads) has a thickness of at least about 1, 5, 10, 20, 30, 40, 50 or 100 microns. In certain preferred embodiments, a gel pad (e.g., in an array of gel pads) has a thickness of less than about 1 millimeter, 500 microns, 200, 100, 50, 40, 30, 20, 10, 5, or 1 microns.

Primers or other reagents can be immobiilized in the gel pads of the gel pad array to prevent migration of the primer out of the gel pad. The immobililzation can be permanent or reversible; covalent or non-covalent.

In another embodiment, an electrical potential can be used to promote a chemical reaction in a gel pad. For example, electrochemical reductive or oxidative cleavage reactions are well known in the art, and can be promoted by application of an appropriate electrical potential to a reaction mixture. Thus, application of a potential to a gel pad can be used to promote an oxidative or reductive reaction in the pad. Any gel pad in an array of gel pads can be selectively targeted for reaction by applying a potential to that gel pad (and its opposed gel pad on the opposing substrate), preferably without subjecting other gel pads in the array to the electrical potential.

In still another embodiment, an electrical potential can be used to promote the migration of a reaction component into a gel pad. Thus, selected gel pads of an array of contacted, opposed gel pads, can be subjected to an electrical potential to promote the migration of reaction components into, or out of, the gel pad (and, preferably, into the opposed gel pad and/or a reaction mixture which surrounds the gel pad).

In still another embodiment, an electrical potential can be used to promote a change in the characteristics of the gel pad. For example, so-called "intelligent gels" have been reported, which are responsive to electrical currents, e.g., the gel shrinks or swells in response to electrical potential. Thus, application of an electrical potential can be used to cause a gel pad to shrink, which could interrupt the electrical current. Thus, a form of feedback control can be attained, e.g., to prevent gel pads from contacting an opposing gel pad, or to maintain opposed gel pads in contact with each other for any desired period of time.

A PCR amplification can then be performed by subjecting the gel pads to thermal cycling as is known in the art. The thermal cycling can be performed with the gel pads in direct contact, or, the gel pads can be separated once the appropriate reaction components have diffused into each gel pad, and each separated gel pad (or array) can be subjected to thermal cycling. In certain embodiments, it is preferred to separate the pads, to

prevent thermal stresses from causing cracking or other loss of integrity of a pad. If desired the gel pads can be brought back into contact after any round of thermal cycling.

During thermal cycling it is preferable to seal the gel pads to prevent evaporation of liquid. Sealing can be provided by placing the gel pads in a hermetically sealed container such as a chamber, or alternatively by covering the gel pad with a non-evaporating liquid such as an oil (which can be removed after cycling, e.g., by washing with a suitable solvent or detergent solution). Between rounds of cycling, the pads can be exposed to fresh reagent solutions, if necessary, e.g., by opening the sealed chamber or by washing away a protective oil layer.

After sufficient rounds of thermal cycling have occurred, the gel pads can be washed to remove excess reagents. The washing step is performed under conditions which do not remove the immobilized (and now extended) primers, but which do remove non-immobilized primers, and other reactants.

The gel pads can now be analyzed to determine a characteristic, e.g., an SNP of the immobilized primers. Either gel pad can be analyzed, or both can be analyzed to provide a redundant analysis (e.g., the analysis of one strand can be compared to the analysis of the other strand to ensure accurate results). A gel pad containing a strand (either target or complement) can also be retained as a backup or for record keeping purposes. In one embodiment, the analysis includes: providing primers which bind adjacent to an SNP, dideoxynucleotides (ddNTPs), and a polymerase (which can be the same polymerase used for the PCR reaction). The ddNTPs are preferably labeled, e.g., with distinct, distinguishable fluorescent labels. The primers are then extended with the polymerase, and the gel pads are washed to remove the unincorporated reactants. The base present at the SNP can then be determined by detection of the labeled ddNTP present in the gel pad.

It will be appreciated from the foregoing that arrays of gel pads can be used, with a first array of gel pads being provided on a first substrate (e.g., a glass plate) and second array of gel pads being provided on a second substrate. The

first and second arrays are preferably prepared in registration, e.g., having the same size, number, and separation of gel pads, so that when the two substrates are brought into close contact, each gel pad of the first array is in contact with a gel pad of the second array.

Microplate Protocol

In the case of detection of polymorphisms in candidate genes, sample, e.g., PCR products, can be distributed to multiple wells, the number depending on the number of polymorphisms in the amplified region to be analyzed - two wells can be for each polymorphism (e.g., 192 biallelic polymorphisms on a 384-well plate). In the case of detection of polymorphisms in a biallelic SNP map, each PCR reaction can be divided between two wells.

An open circle probe could be added to each well. Each allele of each polymorphism should have a separate probe. If both strands were to be analyzed, twice as many probes and twice as many wells would be required. The probes which anneal would be ligated, and then RCA would be performed with labeled dNTPs, preferably two labels, so that both labels are incorporated into the RCA product. The labels might be prompt fluorescence FRET pairs or haptens to which HTRF or LOCI labels could be bound after the RCA. Alleles would be determined by comparing the signals in the two wells containing the two corresponding padlock (circular) probes.

No separation would be required in this assay. A MultiProbe with a thermocycler can be used to automate the liquid handling.

PCR in Gel Pad and RCA Probes for Polymorphism Detection

SNP analysis of a large number of polymorphisms in a biallelic SNP map will sometimes require a large number of amplification reactions. Amplification, e.g., PCR (or NASBA) can be performed in gel pads, with RCA probes. In this

case the probe would be annealed to the immobilized amplification product in the gel pad. The probe design would be relatively simple.

Probes for both (all four) alleles for all polymorphism sites on the array would be applied to the array. the probes would contain allele-specific tags, of which there would be a total of only four - one for each base A, C, G, T. Competing pentamers would not be used, since both (all four) alleles would be present during the hybridization and ligation. The restriction site would not be necessary, In fact, it would be undesirable, since small fragments could diffuse from the gel pads.

There could be only non-fluorescent dNTPs present during the RCA reaction. The RCA products would be labeled with generic allele-specific hybridization probes labeled with different color fluorophors, of which there would be only four (A, C, G, T). The sequences of the allele-specific tags and the probes obviously could be designed to provide very unambiguous differentiation of the four possible alleles (assuming the four fluorescent dyes could be adequately separated). Note that in this case there is great flexibility in the labeling of the probes (compared to the use of fluorescent ddNTP terminators).

Rolling Circle and Additional Amplification

Although RCA (rolling circle amplification) in combination with very sensitive detection or additional round of RCA of signal amplification will often produce measurable signals without amplification, PCR (or some alternative like NASBA) may be desirable for achieving specific detection in some cases, e.g., in some cases of an allele in genomic DNA. Thus, regions of genomic DNA containing sites of polymorphisms can be amplified by PCR prior to contact with circular templates. After PCR the unincorporated primers and dNTPs can be destroyed enzymatically (exonuclease and shrimp alkaline phosphatase). The enzymes would then be destroyed by heating at 80° C.

The TRCA Vector

The TRCA vector is a stretch of nucleic acid sequence designed to contact a nucleic acid in a sample of nucleic acids. In preferred embodiments, the TRCA vector is a single strand of deoxyribonucleotides. In preferred embodiments, the TRCA vector is between 30 and 150 length in length, more preferably between 45 and 130 length and most preferable between 75 and 111 nucleotides in length.

A schematic drawing of a TRCA vector is shown in Fig. 2. In a 5' to 3' orientation the TRCA vector contains the following structurally distinguishable regions as depicted in Fig. 2, a phosphate group, 211, at the 5' end, and a first nucleic acid contacting region, 212, that is complementary to a portion of the nucleic acid fragment in the sample. In specific embodiments, one or more nucleotides in 212 may be designed so as to contain a degenerate (e.g., an equal representation of A, T, G, or C at the position) nucleotide, more specifically at the 5' terminal position of 212 represented by 213. In preferred embodiments, 212 is 25-35 nucleotides long, more preferably 15-20 nucleotides long, most preferable 5-18 bases long. A first spacer segment 214, preferably consisting of 0-16 nucleotides, more preferably of 4-14 nucleotides and most preferably of 8-12 nucleotides.

The TRCA vector also includes a cleavage oligonucleotide target sequence, 220, that has the same 5' to 3' polarity and sequence as the cleavage oligonucleotide. The target sequence 220 is preferably about 15-30 nucleotides long and is, in turn, composed of 5 distinct regions: a unique region, 215, consisting of a variable number of nucleotides, a region 218, containing the recognition site for a type IIs restriction endonuclease,

a region 219, whose length is determined by the cleavage property of the particular type IIs enzyme used and is equal to the number of nucleotides of double strand sequence produced after cleavage.

The region 219 is followed by a sequence tag region, 216, that is preferably composed of 4-8 nucleotides and is more preferably 5 nucleotides

in length. The 5'-most nucleotide of 216 is placed at a fixed displacement from both 218 and 219, so as to position the cleavage site at the phospohodiester bond between the 3'-most nucleotide of 219 and the 5'-most nucleotide of 216.

The TRCA vector also contains a region 217, which has a variable number of nucleotides, and a TRCA primer target sequence, 221, consisting of a variable number of nucleotides. At its 3' end, 221 is bounded by the sequence tag region 216.

The TRCA vector additionally contains a second spacer region, 222, preferably consisting of 0-16 nucleotides, more preferably of 4-14 nucleotides and most preferably of 8-12 nucleotides, and a second nucleic acid contacting region, 223, that is complementary to a region of the nucleic acid that is immediately adjacent to or present at a fixed displacement from hybridizing region of first nucleic acid contacting region, 212, on the fragment in the sample. In specific embodiments, one or more nucleotides in 223 may be designed to contain a degenerate nucleotide(s). In preferred embodiments, 223 is 25-35 nucleotides long, more preferably 15-20 nucleotides long, most preferable 5-18 bases long.

In preferred embodiments, the nucleic acid contacting regions 212 and 223 are designed to contact contiguous bases of a nucleic acid without gaps.

In alternative embodiments, 212 and 213 hybridize to non-contiguous regions of the nucleic acid fragments, in which case a single stranded gap is produced that is bounded by the 5' and 3' ends of the TRCA vector. In specific embodiments these gaps will be filled either by extension by a polymerase in the presence of deoxyribonucleotide triphosphates, or by ligation of short oligonucleotides whose length is equal to the length of the gap.

In other alternative embodiments, the sequence of short oligonucleotides hybridized in the gaps by base stacking can be determined by mass spectrometry.

In additional alternative embodiments, the sequence of short oligonucleotides hybridized in the gaps by base stacking can be determined by hybridization to a Mirzabekov generic chip, or by contacting with a PSBH array.

In additional alternative embodiments, the sequence of short oligonucleotides hybridized in the gaps by base stacking will be determined by contacting with an oligonucleotide ligation array.

In alternative embodiments, the TRCA vector may contain additional segments consisting of a variable number of nucleotides. The additional segments may consist of promoter regions for DNA or RNA polymerases, such as T3 or T7 polymerases, sequences representing recognition or cleavage sites or both for sequence specific nucleic acid binding proteins, ribozymes, splice acceptor or donor sites or both, ribosome binding or translation initiation sites, or any other sequences known in the art that provide a selective advantage or improve the efficiency of the process. Asymmetric sites may be placed either in the same or reverse polarity with respect to the TRCA vector sequence. Additional segments may include nucleic acid sequences that preferentially form hairpins, chi structures, D-loops, cruciform or other specialized structures that provide a selective advantage or increase the overall efficiency of the process.

Oligonucleotides and primers may be partially or wholly double stranded or may be composed partly or wholly of ribonucleotides or a combination thereof. They may be composed of one or more modified nucleotides including, but not limited to, nucleotides such as inosine, 5-methyl cytosine, 7-methyl guanosine, 7-aza guanosine, etc.

In specific embodiments, one or more nucleotides of the TRCA vector or other oligonucleotide or primer may contain modifications at one or

more nucleotide positions. These include, but are not limited to attachment moieties such as biotin, amino or sulfhydryl groups, fluorescent labels, haptens such as 2,4-dinitrophenol or digoxigenin, nucleotide analogs such as peptide nucleic acids (PNA), or other modifications known in the art. The modifications may be attached to the oligonucleotide either directly to the bases, to the phosphate backbone or elsewhere along the length of the oligonucleotide.

Aliphatic or aromatic linkers may be used to attach modifications to the oligonucleotides. The linkers preferably contain between 3-12 repeating monomeric units but may be of any length known in the art. In specific embodiments, the linkers may contain one or more labile groups to serve as release means. The methods are equally adaptable to heat-labile, photo-labile, or chemo-labile, or other release mechanisms known in the art.

Drop-out TRCA Vector

Fig. 3 provides a schematic drawing of a second TRCA vector 520, termed a "drop out vector." The TRCA drop-out vector 520 is a stretch of nucleic acids designed to contact a nucleic acid in a sample of nucleic acids. The vector 520 can be a single strand of nucleotides, e.g., deoxyribonucleotides, ribonucleotides, modified nucleotides, nucleotide analogues or combinations thereof.

Referring to Fig. 3 and proceeding in a 5' to 3' orientation, vector 520 includes a phosphate group 511 located at the 5' end, which is followed by a first nucleic acid contacting region 512. The first nucleic acid contacting region 512 is complementary to a portion of the nucleic acid in a sample. The first nucleic acid contacting region 512 can be, e.g., 20-30 nucleotides, 10-20 nucleotides, or 12-17 nucleotides.

Following region 512 is a first recognition site 513 for a first type IIS restriction enzyme. The length of the first recognition site 513 is determined by the recognition and cleavage properties of the restriction enzyme. The nucleotide cleavage site for the Type IIS enzyme recognizing

the first recognition site 513 is within cleavage site 514, which is located within first nucleic acid contacting region 512. Thus, the first recgonition site 513 is partitioned on the vector such that the 5' most end of the nucleotide cleavage site 514 for the Type IIS enzyme is located within the first nucleic acid contacting region 512. Cleavage site 514 can be, e.g., 10-20 bases from the 5' phosphate group of 512, 8-15 bases from the 5' phosphate group, or 5-10 bases from the 5' phosphate group.

The first recognition site 513 is followed by a TRCA primer target sequence 515. The TRCA primer sequence 515 can be, e.g., 35-40 nucleotides, 25-35 nucleotides, or, 15-25 nucleotides. The TRCA primer target sequence 515 is followed by a second recognition site 516, for a second Type IIS restriction enzyme. The length of the second recognition site 516 is determined by the recognition and cleavage properties of the restriction enzyme. Following the second recognition site 516 is a second nucleic acid contacting region 517 that is complementary to a second portion of a sample nucleic acid. The hybridizing portions of the target nucleic acid are preferably contiguous stretches of nucleotides without gaps. The second nucleic acid contacting region 517 can be, e.g., 20-30 nucleotides, 10-20 nucleotides, or 8-14 nucleotides. In specific embodiments, one or more nucleotides in the second nucleic acid contacting region 517 may be designed so as to contain a degenerate nucleotide, e.g., an equal representation of A, T, G, or C at the portion, more specifically at the 3' terminal position 519 of the second nucleic acid contacting region 517. The 3' most cleavage site 518 for the restriction enzyme 2 is adjacent to the 3' most nucleotide of region 517. The 3' most nucleotide of 518 is placed at a fixed displacement from recognition site 516, so as to position the 3' restriction enzyme cleavage site at the phosphodiester bond at the 3' most nucleotide of the second nucleic acid contacting region 517. Thus, cleavage at the first restriction enzyme site 514 and at the second restriction enzyme site 518 will generate, or "drop-out", a nucleotide fragment 512 containing a portion of the target sequence. The excised nucleotide fragment 512 can be, e.g., 10-20 nucleotides, 8-15

nucleotides, or 5-10 nucleotides long. The first and second restriction enzymes can be the same or different enzymes. In alternative embodiments, the TRCA "drop out" vector is in an inverse polarity, i.e, is in a 3' to 5' orientation relative to the 5' to 3' orientation shown in Fig. 3.

TRCA excision vector

Another type of TRCA vector, termed a TRCA excision vector, is shown schematically in Fig. 4. The TRCA excision vector 620 allows for excision of a target sequence and is a stretch of nucleic acids designed to contact a nucleic acid in a sample of nucleic acids. The TRCA excision vector 620 can be a single strand of nucleotides, e.g., deoxyribonucleotides, ribonucleotides, modified nucleotides, nucleotide analogues or combinations thereof. As is shown in Fig. 4, in a 5' to 3' orientation, the TRCA excision vector 620 contains a phosphate group 611 at the 5' end and a first nucleic acid contacting region 612 that is complementary to a portion of nucleic acid in a sample.

In specific embodiments, one or more nucleotides in the first nucleic acid contacting region 612 may be designed so as to contain a degenerate nucleotide, e.g., an equal representation of A, T, G, or C at one or more nucleotides, e.g., at the 5' terminal position 613 of the first nucleic acid contacting region 612. The first nucleic acid contacting region 612 can be, e.g., 25-35 nucleotides, 15-20 nucleotides long, or 5-18 bases.

The first nucleic acid contacting region 612 is followed by region 614, which is complementary to a TRCA primer sequence. The TRCA primer sequence can be, e.g., 35-40 nucleotides, 25-35 nucleotides, or 15-25 nucleotides. In some embodiments the complementary TRCA primer has a 5' non-complementary region that creates a 5' non-complementary tail, e.g., as indicated in the TRCA primer 114 of Fig. 1.

Region 614 is followed by a spacer 615 that is composed of, e.g., 0-8 nucleotides. Adjacent to the spacer 615 is a sequence tag region 616 that is, e.g., 4-10 nucleotides or 5 nucleotides. Sequence tag region 616 is followed by a restriction enzyme recognition site 617, whose length is determined by the cleavage and recognition properties of the particular restriction enzyme. The cleavage site 618 for the restriction enzyme is contained at the 3' end of the restriction enzyme recognition site 617, so that cleavage will result in generation of a fragment most preferably 5-10 nucleotides in length containing the sequence tag region 616 at its 3' end.

Following region 618 is a second nucleic acid contacting region 619 that is complementary to a portion of the nucleic acid in a sample. The second nucleic acid contacting region 619 can be, e.g., 25-35 nucleotides, 15-20, or 5-18 nucleotides.

In alternative embodiments, the TRCA excision vector is in an inverse polarity, i.e, is in a 3' to 5' orientation relative to the 5' to 3' orientation shown in Fig. 4.

The TRCA excision vector 620 allows for excision of a target sequence upon digestion with a restriction enzyme recognizing the enzyme recognition site 617.

Thermophilic Rolling Circle After Cleavage with Endonuclease (TRACE) Vector

Fig. 5 illustrates a schematic drawing of a TRACE vector, for thermophilic rolling circle amplification after cleavage with endonuclease. The TRACE vector 815 is composed, in a 5' to 3' orientation, of a first unique region 811 that is complementary to a target nucleic acid in a sample. The first unique region 811 can be, e.g., 25-35 nucleotides, 15-25 nucleotides, or 5-15 nucleotides in length.

The first unique region 811 is connected to a first spacer region 825. The first spacer region 825 can be, e.g., 10-15, 5-10, or 0-5 nucleotides. Located to the 3' end of the first spacer region 825 is a first paring region 812, which includes a recognition sequence 813. Located to the 3' end of the first pairing region is a first spacer element 214, which is also indicated as 814 in the Fig. 5, and elements 215-222 as described above and illustrated in Fig. 2. Located to the 3' end of the second spacer region 222, also indicated as 816 in Fig. 5, is a second recognition sequence 817. A double stranded region generated by complementary base pairing, preferably without gaps, between regions 812 and 817 creates a recognition sequence 813 for a restriction means, e.g., an endonuclease, a restriction endonuclease, e.g., a Type IIS restriction endonuclease. The recognition site 813 is positioned to produce cleavage at the junction of 812 and 814 on one strand of the duplex.

Located on the 3' side of the second recognition sequence 817 is a second spacer region 826 which is preferably not complementary to the first spacer region 825. Second spacer region 826 is, e.g., 10-15 nucleotides long, more preferably 5-10 nucleotides long and most preferably 0-5 nucleotides long.

The second spacer region 826 is connected on its 3' end to a second unique region 819 that is complementary to a target nucleic acid in a sample. The second unique region 819 can be, e.g., 25-35 nucleotides, 15-25 nucleotides, or 5-15 nucleotides in length.

The first and second spacer regions 825 and 826 are preferably not complementary nucleotides in the target nucleic acid fragment between regions contacted by unique regions 811 and 819.

The ligation template oligonucleotide 820 is comprised, in order, of a first region 821 complementary to a part of region 215 of the TRACE vector 815. First region 821 can be, e.g., 10-20 nucleotides, 8-15 nucleotides, or 5-10 nucleotides long. First region 821 is followed by second region 822 that is complementary to region 214. Second region 822 is in turn followed

by third region 828, which is complementary to a sequence 827 on the TRACE vector between elements 222 and 817. On the 3' end of third region 828 is fourth region 823, which is complementary to region 221 of the vector 815.

Ligation template oligonucleotide 820 is thus designed so that after double strand cleavage of vector 815 by a recognition means specifically recognizing 813, the resulting cleaved ends include a phosphate group at the 5' terminal nucleotide of 814 and a hydroxyl group at the 3' terminal nucleotide of 827. These ends are juxtaposed by hybridization without gaps due to sequence complementarity to regions 822 and 828, respectively. As a result, they are targets for ligation by a ligase, such as a DNA ligase. Thus, ligation template oligonucleotide 820 is opposite in polarity to the 5' to 3' orientation of the vector 815.

TRCA Primers

The structure of one TRCA primer is depicted in Fig. 6. The TRCA primer consists of a variable number of nucleotides and is preferably 26 to 45 nucleotides. The TRCA primer includes at least 3 functionally distinct segments. The presence of the 5' most segment, 311, is optional. The 311 segment is composed of 8-15 nucleotides whose sequence is non-complementary to any contiguous region of the TRCA vector or other nucleic acids used in the TRCA process. The 5' end of the TRCA primer preferably lacks a phosphate group but may optionally contain a capture moiety such as biotin, amine-, sulfhydryl-, succinimidyl, or other moieties. These moieties are preferably attached to the primer oligonucleotide by a linker, optionally a cleavable aliphatic linker. Segment 312 is preferably composed of 18-30 nucleotides that are complementary to a contiguous region of the RCA vector. Segment 313 includes the terminal 6-8 nucleotides of the TRCA primer that are complementary to the sequence tag region, 216. 313 is capable of serving as an extension initiation site for a polymerase when correctly hybridized to

the TRCA vector. Additionally, the TRCA primer may contain one or more additional segments of a variable number of nucleotides, particularly at or near its 3' end. The TRCA primer may contain alternate or modified bases.

Cleavage Oligonucleotide

The cleavage oligonucleotide is a single strand oligonucleotide capable of hybridization to polymerase extension products arising from the procedure shown in `Fig. 1. The cleavage oligonucleotide specifically hybridizes to the extension products and thus provides a partially double stranded region or regions on the concatamer. The double stranded region includes, in order, the restriction endonuclease recognition site, the number of nucleotides present between the recognition and cleavage sites of the type IIs re and cleavage site, as well as additional flanking nucleotides.

A schematic drawing of a cleavage oligonucleotide is shown in Fig. 7. The cleavage oligonucleotide includes a first spacer region, 411, preferably of 5-12 nucleotides, a restriction enzyme recognition site, 4.12, whose length is specified by the property of the restriction enzyme used, preferably in the range of 4-6 nucleotides, a segment 4.13 whose length in nucleotides is equal to the number of bases of double-stranded region between the restriction enzyme recognition and cleavage sites, a region 4.14, whose sequence is complementary to the sequence tag site, 216 in the TRCA vector, and a second spacer region, 4.15, consisting of a variable number of nucleotides, preferably 5-12 nucleotides.

In preferred embodiments, the 5' end of cleavage oligonucleotide 4.10 is not phosphorylated and the 3' hydroxyl moiety is blocked by a chemical moiety, 4.16, such as phosphorylation, 3'-O-methyl modification or other method known in the art.

In alternative embodiments the cleavage oligonucleotide is comprised of two short oligonucleotides stabilized by base stacking on the

TRCA linear products. A schematic drawing of the two part cleavage oligonucleotide is shown in Fig. 8. The 5' oligonucleotide 711 can be, e.g., 5-12 nucleotides, and contains a restriction enzyme recognition site 712 whose length is specified by the property of the restriction enzyme used, and can be, e.g., in the range of 4-6 nucleotides. A spacer region of 1-14 nucleotides 713, follows restriction recognition site 712. The second oligonucleotide 714, contains a sequence tag of preferably 5-8 nucleotides in length. Hybridization of oligonucleotides 711 and 714 to a TRCA linear product 716 creates a double-stranded cleavage site containing a single stranded nick. The recognition site 712 is approximately positioned on 5' oligonucleotide 711 so that the single strand nick is located at the junction of oligonucleotides 713 and 714. Cleavage of the duplex creates fragment 719 containing the 5-8 nucleotide sequence tag on the 3-end of the DNA.

Capture oligonucleotide

Capture and arraying of oligonucleotide capture probes using the Mirzabekov generic chip hybridization are known in the art. Capture and arraying of oligonucleotide capture probes using the positional sequencing by hybridization (PSBH are also known in the art.

Enzymes and proteins

The various enzymes described herein are either commercially available or may be prepared by methods know in the art. These include ligases, polymerases, restriction endonucleases, ribonucleases, RNase H, other DNA modifying enzymes, redox enzymes, b-lactamase, metal binding proteins, green fluorescent protein, proteases, and kinases. Enzymes may also be employed for tagging nucleic acids, detection of signals, or as anchors.

Other suitable proteins are those that have affinity to or bind to DNA. These include, but are not limited to, Ssb, recA or its homologues, or

other proteins that associate with DNA such as transcription or replication factors, or those that form complexes with proteins that exhibit properties of association with DNA.

This methods described herein are equally adaptable to the use of chemical entities that associate with DNA either in a sequence-specific or unspecific manner, including chemicals such as pyrylium iodide (P2), propidium iodide (PI) ,ethidium bromide, TOTO, YOYO, Sybr Green, neocarzinostatin, cisplatin, bleomycin, etc.

Formats and Devices

Microplate formats

TRCA assays are preferably performed in microplates, preferably in 96-well microplates, more preferably in 384-well plates, most preferably in 1536-well plates.

Acrylamide Gel Pad Arrays

TRCA assay products can be partitioned by specific hybridization and/or ligation to nucleic acid tags within acrylamide gel pad arrays. In addition, TRCA assays can be miniaturized to allow TRCA and detection to occur simultaneously on the gel pad arrays.

The methods described herein can be performed in single tubes, on surfaces such as glass, silica matrices, cellulose, nitrocellulose, nylon, or aluminum oxide membranes, acrylamide, polystyrene, polypropylene, vinyl acetate, polymethacrylate, polyethylene, polyethylene oxide, polycarbonates, polyesters, polypropylfumarates, polyglycollic acid, polyanhydrides, glycosaminoglycans, polyaminoacids, silicon rubber, agarose, latex, silicon dioxide, fluorocarbons, metal supports, teflon, plastic, or other surfaces known in the art.

The surfaces can have any form including, but not limited to, three-dimensional pads such as gel pads, gel pad arrays, wells or microwells, porous structures such as channels, microchannels, beads or microparticles. The preferred forms of the substrate include microtiter dishes, three dimensional acrylamide or agarose gel pad arrays, and porous membranes. The methods are equally adaptable to other forms of the substrate such as thin films, bottles, dishes, fibers, or shaped polymers.

One more components of the TRCA process may be immobilized in one or more microwells or elements of a gel pad array. These components include, but are not limited to, components such as the target nucleic acid, oligonucleotides such as, TRCA primer, cleavage oligonucleotide, oligonucleotides that interrogate the sequence of the sequence tags of TRCA vectors, or capture oligonucleotides, singly or in combination.

In a preferred embodiment, the TRCA process is performed in the absence of cleavage steps. In this implementation a single species of a TRCA primer is immobilized in a well of a microplate.

Multiplex 1.

The TRCA reaction mixture containing the target, enzymes, oligonucleotides and primers, and other components of the TRCA process are incubated in a microwell. Following circle closure by the action of a ligase, extended concatameric single stranded nucleic acids are anchored in the microwell. The extended products are quantitated. In preferred embodiments, detection and quantitation of the signals generated are preferably performed by a homogeneous assay, preferably a beacon assay.

In alternative embodiments, the TRCA process includes a cleavage step. In this embodiment capture probe or probes are immobilized in the microwell in addition to the TRCA primer. Signals generated by interactions of the cleaved fragments are either spatially resolved by

positional features of the capture probes or spectrally resolved by generating a separate signal for each capture event and quantitated. A plurality of capture probes immobilized in each microwell or gel pad capture a plurality of signals or potential signals generated in the pad by sequence encoding.

An example of sequence encoding is herein described in specific relation to signals generated in a single gel pad or microwell by a cleaved fragment containing a sequence tag, 3' GTATC -5'. For this implementation, the gel pad or microwell contains 4 immobilized capture probes that contain the 3' termini,

5' N1N1N1N1N1N1N1N1N1N1N1N1N1N1CATAG 3'

Capture probe 1

5' N2N2N2N2N2N2N2N2N2N2N2N2N2N2AATAG 3'

Capture probe 2

5' N3N3N3N3N3N3N3N3N3N3N3N3N3N3TATAG 3'

Capture probe 3

5' N4N4N4N4N4N4N4N4N4N4N4N4N4N4GATAG 3'

Capture probe 4

In this form of the capture probe, the 5'-most region of the capture probe depicted by the stretch of N1 nucleotides, the invariant region, is specific to capture probe 1. It is important to note that the invariant region is not a homopolymeric stretch of nucleotides but can be composed of any sequence of nucleotides. The nucleotide sequence of the invariant region is preferably not homologous to any sequence of contiguous nucleotides in the TRCA vector.

Cleaved fragments are interrogated by specific hybridization of the 3' terminal sequences of cleaved fragment, namely the sequence tag

region to the 3' terminal 5 bases of the capture probe as shown. Specifically, the 3' terminal nucleotide contained in the cleaved fragment, namely G in this example, is interrogated by the 5th nucleotide from the 3' end of the capture oligonucleotide. An additional short oligonucleotide, the reporter oligonucleotide, whose sequence is complementary to sequence of the invariant region of capture oligonucleotides also hybridizes, such that the 5' terminal nucleotide of the reporter oligonucleotide is in register with the 6th nucleotide of the capture probe. In this configuration, the cleaved fragment and the reporter oligonucleotide are hybridized adjacent to each other without gaps on the capture probe and hybridization of the reporter oligonucleotide is stabilized by base stacking or ligation. The four distinct capture probe sequences hybridize to four distinct reporter oligonucleotides. Each of the reporter oligonucleotides is tagged with a distinct detection moiety. Sequence encoding is then achieved by the specific signal detected in the gel pad. In the example cited above, a single signal due to capture probe 1 is detected, which corresponds to the specific detection moiety tagged to reporter oligonucleotide 1 that hybridizes to capture

probe 1.

Arrays can be analyzed using mass spectrophotometry, in which is based on analysis of oligos hybridized in gap;

Mirzabekov analyses, in which sequence analysis of oligos hybridized in gap by base-stacking hybridization to generic chip;Cantor/PSBH analyses, which rely on oligonucleotide ligation, e.g., gap-filling or double ligation.

Detection

Several methods of detection can be employed.

Fluorescent Labels

In active labeling, DNA generated from TRCA is labeled directly during the DNA polymerization step by incorporation of a fluorescently

labeled deoxynucleotide using any of the methods known to those skilled in the art. The unit length product of the TRCA reaction contains a known number of the deoxynucleotides to be labeled such that the number of unit length products can be quantitated by direct fluorescence measurements.

In passive labeling, DNA generated from TRCA is labeled passively by hybridization to a labeled oligonucleotide containing sequences complementary to either the TRCA products themselves or to a capture oligonucleotide. Passive oligonucleotides are labeled using fluorescence, haptens, or proteins using any of the methods known to those skilled in the art. Each unit length TRCA product will react directly or indirectly with one labeled passive oligonucleotide allowing direct quantitation.

Probe nucleotides can alternatively be labeled using FRET nucleic acid probes. Oligonucleotides labeled on the 5' or 3' end, or two separate oligonucleotide probes with FRET pair indicators, can be used to detect a target TRCA product sequence through energy transfer upon DNA hybridization. FRET pair molecules can be placed on the 5' and 3' ends of labeling oligonucleotides can be commercially synthesized using methods known to those skilled in the art.

In RNase protection, hybridization of an RNA containing FRET pair oligonucleotide to unit length TRCA products allows protection of the RNA containing probe from RNase digestion. Protection of RNA containing probe allows protection of the FRET pair and prevents loss of fluorescence transfer in the presence of RNase.

Yet another method of detection utilizes FRET-Quench nucleic acid probes. With RNase H detection, hybridization of an RNA containing FRET-Quench oligonucleotide to unit length TRCA products allows cleavage of the RNA containing probe by RNaseH. Cleavage of the RNA-DNA duplex results in fluorescence increase of the donor molecule.

Alternatively, hybridization of a FRET-Quench oligonucleotide containing a restriction enzyme recognition and cleavage site to the TRCA

products allows cleavage of the TRCA product-oligonucleotide hybrid. Cleavage of the FRET-quench oligonucleotide thus results in fluorescence increase of the donor molecule.

Detection can alternatively occur using methods based on reversible protein interaction, disulfide bond formation, or appropriate redox labels. For example, NADH oxidase: Thiol end labeled passive oligonucleotides disulfide linked NADH oxidase allows direct detection of electron production followed by the reduction of di-oxygen to hydrogen peroxide. Electrochemical measurements of the redox reaction allows detection of the interaction between the enzyme labeled passive oligonucleotide and the TRCA products.

Alternatively, morphinone reductase and thiol end labeled passive oligonucleotides disulfide linked to morphinone reductase allows detection via electron transfer concomitant with the reduction of NADH using codeinone as the oxidizing substrate. Electrochemical measurements of the redox reaction allows detection of the interaction between the enzyme labeled passive oligonucleotide and the TRCA products.

Suitable chromophoric substrates can include bð-lactamase. Thiol end labeled passive oligonucleotides disulfide linked to a bð-lactamase allows detection of a passive oligonucleotide via cleavage of a chromophoric substrate (i.e. nitrocefin). Cleavage of the bð-lactam ring results in a change in the absorbance maxima of the chromophore. An increase in the absorbance maxima of the cleavage product is an indicator of an interaction between a bð-lactamase linked passive oligonucleotide and TRCA products.

Fluorescent substrates can utilize the action of proteases. Thiol end labeled passive oligonucleotides disulfide linked to an any protease (i.e., Ser, Cys, Lys, Metallo, caspase proteases) allows detection via cleavage of a peptide substrate containing a FRET-Quench pair on its termini. Cleavage of the peptide thus results in fluorescence increase of the donor molecule.

bð-ðlactamase ð ðbð-lactamase activity can also be detected by a substrate molecules that changes from green to blue when cleaved (due to disruption of FRET) (Zlokarnik et al., 1998). The change in emission ratio reveals interaction between a bð-lactamase linked passive oligonucleotide TRCA products.

Thiol end labeled oligonucleotides disulfide linked to carbonic anhydrase allows detection of a passive oligonucleotide via a pH sensitive fluorophore, pyranine (Shingles and Moroney, 1997). Production of CO_2 changes the pH of the solution and increases the fluorescence of the indicator.

Thiol end labeled oligonucleotides disulfide linked to a protein kinase (Kinase C Casein kinase, Tyrosine kinase, MAP kinase) allows detection of the oligonucleotide via cleavage of a FRET-quench pair labeled substrate causes an increase in the fluorescence of the donor molecule.

Detection methods can also be based on metal binding proteins. For example, in the Zinc binding/metallothionein system, the 20 Cys residues in metallothionein allows binding of seven Zn2+ per enzyme molecule. Addition of a reducing agent to the complex of the thiol end labeled passive oligonucleotides disulfide linked to a 7:1 metallothionein:Zn2+ complex results in release of the bound Zn2+ (Maret and Vallee, 1998). Quantitation of free Zn2+ with an indicator dye allows quantitiation of the interaction between the passive oligonucleotide and the TRCA amplification.

In calcium binding/calmodulin-based systems, thiol end labeled passive oligonucleotides disulfide linked to fluorescently labeled calmodulin can be detected by Ca+ induced changes in protein fluorescence (Blair et al., 1994). Alternatively, bound unlabeled calmodulin can be detected using a Ca2+ luminescent analog such as a lanthanide (Hill et al., 1994) or quinoline indicator (Tatsumi et al., 1997). Since one protein will bind per interaction between the passive oligonucleotide and a TRCA product, any of these methods will allow quantitiation of the TRCA amplification.

An additional detection system is based on Green Fluorescent Protein/Green Fluorescent Protein tags (GFP). Thiol end labeled passive oligonucleotides disulfide linked to GFP and/or GFP-tagged proteins allows direct fluorescence detection of the interaction between the passive oligonucleotide and the amplified TRCA products.

Alternatively, oligonucleotides directly labeled with haptens (digoxigenin, i.e. biotin) can be detected using both chemiluminescence and enzyme linked antibodies using methods know to those skilled in the art.

TRCA nucleic acid products can additionally be detected using intercalating agents such as but not limited to pyrylium iodide (P2), propidium iodide (PI) , ethidium bromide, TOTO, YOYO, Sybr Green, neocarzinostatin, cisplatin, bleomycin, using methods known to those skilled in the art.

Wave Guides

Using a two-dimensional optical wave guide and light scattering labels hybridization patterns on PBSH arrays can be detected (Abbott Labs, Stimpson et al., 1995). An array detection oligonucleotide contains a label recognized by an antibody conjugate linked to a large light scattering atom such as selenium. The evanescent wave created by the wave guide scatters light from a particular source allows measurement of only the bound oligonucleotide.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1

Heat Denaturation of Biotin Labeled Target Nucleic Acid

Biotin labeled nucleic acids are immobilized on streptavidin beads (Dynal, Lake Success, NY) in a microtiter well containing 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1.0 mM NaCl according to manufacturer's instructions. The nucleic acids are heat denatured at 95 degrees C for 5 minutes. The sample is chilled on ice for 5 minutes and then placed on a magnet for 2-3 minutes until a tight pellet of beads is formed. The beads are washed with buffer containing no salt. The supernatants are discarded. The single-stranded biotin labeled nucleic acid will remain in the microtiter well.

Example 2

Denaturation of Unlabeled Target Nucleic Acids

Nucleic acids are heated to 90 degrees C for 5 minutes the quick chilled on ice for 2-3 minutes prior to use. Nucleic acids can also de denatured using sodium hydroxide prior to use.

Example 3

Structure of TRCA Probe

A TRCA vector oligonucleotide is prepared having the sequence 5'(PO4)-CTTCTGAGGACTCTATAGCACTAGCATGGGAUG-TTATAGATCAATCA TGTCAGGATTACGATACGCACTAGATAAGTTCT-3' (OH). Upon hybridization to target nucleic acid, the 5' and 3' ends are positioned adjacent to each other as a result of the 15 nucleotides on the 5' end and the 15 nucleotides on the 3' end of the TRCA vector being complementary to target nucleic acid sequence. The oligonucleotide has a BstF51 restriction enzyme cleavage site and a deoxyuracil (dU) in place of the thymidine nucleotide in the enzyme recognition site. The restriction enzyme recognition site is placed at a fixed position relative to the sequence tag on the vector. This ensures that, upon cleavage, the coding sequences in each TRCA vector is located at

the 5' end of the cleaved product, facilitating its interrogation by the arrayed oligonucleotides.

Example 4

Structure of a Rolling Circle Amplification (RCA) Primer

An RCA primer is prepared having the sequence 5'-TCGTAATCCTGACATGATTGATCTATAA -3'(OH). The 5' end is unphosphorylated. There is a five nucleotide allele specific sequence tag (TATAA) on the 3' end of the primer.

Example 5

Structure of a Rolling Circle Amplification (RCA) Primer

With an Additional 5' Sequence

An RCA primer is prepared having the sequence shown in Example 4, and having the additional sequence 5'-ATCCACTA at its 5' end.

Example 6

Structure of Cleavage Oligonucleotide

A cleavage oligonucleotide has the sequence 5'-CTAGCATGGGATGTTATAGATCAAT -3'. The 5' end is unphosphorylated and the 3' OH is blocked to prevent extension or ligation of the oligonucleotide. The cleavage oligonucleotide anneals to the sequence in the growing concatameric strand providing a double stranded BstF51 restriction enzyme recognition and cleavage site. Following enzymatic digestion, the short cleaved products from the cleavage oligonucleotide dissociate by melting from the cleavage products. The complementary, unit length, amplified products generate a fragment containing the sequence 5'-

TTATA-3' at the end of the fragment. This is the complementary sequence to the five nucleotide allele specific marker (TATAA) at the 3' end of the RCA primer described in Examples 4, and 5, above.

Example 7

Structure of Capture Oligonucleotide Probe

A capture oligonucleotide contains the sequence 5'-CTGAATTGCTAGCTGTTAGTCGCGCTTAGT-AATAT-3' such that the five 3' bases are complementary to the 5 base tag sequence (5'-ATATT-3') present in the amplified products generated from polymerization from the TRCA oligonucleotide in Example 4. The 5' end of the oligonucleotide is linked to a gel pad array, while the 3' end is available for hybridization.

Example 8

Structure of Array Detection Oligonucleotide

A capture oligonucleotide is constructed containing the sequence 3'-GACTTAACGATCGACAATCAGCACGAATCA-5' such that it contains sequences complementary to the 5' 15 bases of the capture probe described in Example 7.

Example 9

Circularization of TRCA Vector by Hybridization/Ligation to a Target Nucleic Acid

A ligation reaction is prepared containing 15 nM single-stranded target nucleic acid, 150 nM TRCA probe, 0.5 mM NAD, 10 mM MgCl2 0.01% Triton X-100, and 25 units Ampligase (Epicentre Technologies, Madison, WI) thermostable DNA ligase in 20 mM Tris-HCl buffer (pH 8.3),

in a total volume of 50 mõl. The reaction is heated to 90 degrees C for 5 minutes then incubated at 60 degrees C for 1 hour.

Example 10

Primed Amplification of a Circularized TRCA Probe

To the ligation reaction from Example 9 is added 25 mõl of mixture containing 20 mM Tris-HCL (pH 8.3), 0.01% Triton X-100, 30 mM KCL, 3 mM each dATP, dTTP, dCTP, dGTP (one of which is fluorescently labeled), 3 mõM RCA primer, 15 % DMS0, 30 mõM T4 gene-32 DNA binding protein (Amersham Life Sciences, Piscataway, NJ) and 10 units Vent (exo-) DNA polymerase (New England Biolabs, Beverly, MA). The reaction is heated to 90 degrees C for 5 minutes then incubated at 60 degrees C for 120 minutes. Unincorporated dNTPs are removed by centrifugation using a Sephadex G50 minicolumn (Boehringer Mannheim, Indianapolis, Indiana). Incorporation of fluorescent nucleotides into concatameric DNA is quantitated by direct fluorescence measurements and/or by agarose gel electrophoresis.

Example 11

Detection of Amplified TRCA Products Using Direct Fluorescence Energy Transfer (FRET) from Two Oligomers

FRET DNA oligonucleotides labeled with a 5' fluorescence donor (Fluorescein) and a 3' acceptor (rhodamine) are purchased commercially (Biosynthesis, Inc., Lewisville, TX). To the rolling circle amplification reaction in Example 9, a final concentration of 10 mõM of each fluorescently labeled separate FRET oligomers with Tm > 60 degrees C (125 and 126) are added the reaction, which is then heated at 90 degrees C for 3 minutes. The reaction is placed at 60 degrees and monitored by the increase in the donor fluorescence emission upon hybridization of the FRET pair oligonucleotides.

Example 12

Detection of Amplified TRCA Products Using Thermophilic RNase H and Fluorescence Energy Transfer (FRET)

The cleavage oligonucleotide described in Example 6 is labeled with a 5' fluorescence donor (Fluorescein) and a 3' quencher (3-nitrotyrosine). To the TRCA reaction in Example 10, a final concentration of 10 mõM of an FRET-Quench an alternate version of the cleavage oligonucleotide described in Example 6 is added the reaction, which is then heated at 90 degrees C for 3 minutes. This cleavage oligonucleotide possesses the same nucleotide sequence except the 5 GGGATG 3' sequence is replaced by ribonucleotides (5'GGGAUG 3'). The reaction is placed at 60 degrees C with 10 units ribonuclease H from Thermus thermophilus HB8 (Itaya et al., 1991). Upon hybridization of the cleavage oligonucleotide the reaction can be monitored by the increase in the fluorescence emission upon cleavage of the RNA region by RNase H.

Example 13

Cleavage of Double-Stranded TRCA Products Using a Site-Specific Restriction Endonuclease (RE)

To the TRCA reaction in Example 9, a final concentration of 10 mõM of the restriction enzyme cleavage oligonucleotide (Example 6) is added to the reaction, which is then heated at 90 degrees C for 3 minutes. The reaction is placed at 65 degrees C and 10 units of BstF51 (New England Biolabs, Beverly MA) restriction enzyme is added. The reaction is incubated for another 120 minutes at 60 degrees C. Cleavage products are analyzed directly by polyacrylamide gel electrophoresis and staining with Sybr-Green II dye (Molecular Probes, Eugene, OR). Unincorporated dNTPs are removed

and the cleaved amplified DNA by direct fluorescence as described in Example 14.

Example 14

Detection of RE Cleaved TRCA Products Using

A FRET-Quench Oligonucleotide

To the TRCA reaction described in Example 10, a final concentration of 10 mδM of a FRET-Quench labeled restriction enzyme cleavage oligonucleotide (Example 6) is added. The reaction is then heated at 90 degrees C for 3 minutes. The reaction is then placed at 65 degrees C and 10 units of BstF51 (New England Biolabs, Beverly MA) restriction enzyme is added. The reaction is incubated for another 120 minutes at 60 degrees C. The amplified DNA is quantitated by the increase in the fluorescence donor emission upon cleavage of the double-stranded DNA region by the restriction endonuclease. TRCA products are detected using any of the method described in Example 10.

Example 15

Rolling Circle Amplification Concomitant with Ligation and Restriction Endonuclease Cleavage: Thermophilic RCA

For concomitant ligation, amplification, and cleavage 10 nM single-stranded target nucleic acid, 100 nM TRCA vector, 1 mδM RCA primer (unphosphorylated), 10 mδM of cleavage oligonucleotide (unphosphorylated), 0.5 mM NAD, 10 mM MgCl2, 10 mM KCL, 0.01 % Triton X-100, 1 mM each dATP, dTTP, dCTP, dGTP, 5 % DMS0, 10 mδM T4 gene-32 DNA binding protein, 25 units Ampligase thermostable DNA ligase, 10 units Vent (exo-) DNA polymerase, and 10 units of BstF51 restriction enzyme in 20 mM Tris-HCL buffer (pH 8.3) are heated to 90

degrees C for 2 minutes then incubated at 60 degrees C for 120-240 minutes. Cleaved TRCA products are detected using the method described in Example 11.

Example 16

Rolling Circle Amplification Concomitant with Ligation

And Thermophilic RNaseH Cleavage

For concomitant ligation, amplification, and cleavage, 10 nM single-stranded target nucleic acid, 100 nM TRCA probe, 1 mδM RCA primer (unphosphorylated), 10 mδM of RNaseH cleavage (1.27) FRET-Quench oligonucleotide (unphosphorylated), 0.5 mM NAD, 10 mM MgCl2, 10 mM KCL, 0.01 % Triton X-100, 1 mM each dATP, dTTP, dCTP, dGTP, 5 % DMS0, 10 mδM T4 gene-32 DNA binding protein, 25 units Ampligase thermostable DNA ligase, 10 units Vent (exo-) DNA polymerase, and 10 units of thermophile Thermus thermophilus HB8 RnaseH in 20mM Tris-HCL buffer (pH 8.3) are heated to 90 degrees C for 2 minutes then incubated at 60 degrees C for 120-240 minutes. The amplified DNA is analyzed using the method described in example 12.

Example 17

Array Detection of Restriction Enzyme Cleaved TRCA Products Using a Fluorescently Labeled Detection Oligonucleotides

Flourescently labeled array detection oligonucleotides (Example 8) are purchased commercially (i.e, Biosynthesis, Lewisville, TX). Restriction-enzyme cleaved TRCA products containing a tag sequence at the end of the single-strand nucleic acid are partitioned on a acrylamide gel pad array containing the array oligonucleotide and the capture probe described in Example 8. Hybridization of the five nucleotide tag sequences present on the

3' end of the capture probe and the 3' end of the RE cleavage product is detected by ligation between the RE cleaved TRCA fragments and the array detection oligonucleotide described in Example 8. The ligated array detection oligonucleotides is visualized and quantitated using direct fluorescence.

Example 18

Array detection of Restriction Enzyme Cleaved TRCA Products Using a Hapten Labeled Detection Oligonucleotides

Array detection oligonucleotides (Example 8) are synthesiszed commercially possessing haptens such as digoxigenin or biotin at either the 5' or 3' end (i.e., Biosynthesis, Inc., Lewisville, TX). Restriction enzyme cleaved TRCA products are arrayed using the method described in Example 17. Hapten labeled array detection probes are visualized using direct and enzyme-linked methods.

Example 19

Crosslinking a 3'SH Oligonucleotide to a Detection Protein By Disulfide Bond Formation

Thiol labeled oligonucleotides are obtained from a commercial vendor (Biosynthesis, Inc., Lewsiville, TX). Disulfide bond formation is detected by detecting the SH-labeled oligonucleotide.

Example 20

Detection of a SH labeled-Oligonucleotide Using the Zinc Binding Protein Metallothionein

Metallothionein (MT) is prepared from rabbit or human, reduced, and reconstituted with the zinc such that 7 zinc molecules are bound to the 20 Cys residues as described(Maret and Vallee, 1998). Bound zinc is quantitated using atomic absorption spectrophotometry. The MT-zinc complex is reacted with the 3'SH oligonucleotide to allow disulfide bond formation as described in Example 19. Unlabeled oligonucleotide is removed by size exclusion chromatography. Excess MT-zinc-oligonucleotide is reacted on the PBSH array such that ligation of the 3' end of the TRCA amplified target nucleic acid with the 5' end of the labeled passive oligonucleotide occurs. Excess MT-zinc-oligonucleotide is removed and zinc is released from the MT-oligonucleotide using a reducing agent such as dithiodipyridine. Free zinc is quantitated using a zinc-complexing dye such as 4-(2-pyridylazo)resorcinol (PAR; eð500nm = 65,000 M-1 cm-1 at 500 nm or 2-carboxy-2 -hydroxy-5 sulformazylbenzne (Zincon, ðeð620nm = 17,500 M-1 cm-1.

Example 21

Detection of a SH Labeled-Oligonucleotide Using

Calcium Induced Conformational Changes

In Fluorescein Labeled Calmodulin (F-CaM)

Purified F-CaM (Sigma) is reacted with the 3'SH oligonucleotide as described in Example 19. Calcium (Ca+) is added in 10-fold excess to the reaction. Free Ca2+ is removed. Ca2+ bound to CaM causes conformation changes in the F-CaM-oligonucleotide which are detected is a fiber optic sensor method described by Blair et al., 1994.

Example 22

Detection of a SH Labeled-Oligonucleotide Using Lanthanide Binding to Calmodulin (CaM)

Purified CaM (Santa Cruz Biotechnologies, Santa Cruz, CA) is reacted with the 3'SH oligonucleotide as described in Example 19. Calmodulin-oligonucleotide is detected using the lanthanide TBCl3 (Aldrich). Excess lanthanide is removed by washing and calmodulin-bound lanthanide is detected by excitation using a Xenon lamp from 270-385nm for TB3+ or 320-385 for Eu3+. Emission is monitored above 515nm.

Example 23

Detection of a SH Labeled-Oligonucleotide Using Quin2 Binding to CaM

Purified CaM (Santa Cruz Biotechnologies, Santa Cruz, CA) is reacted with the 3'SH oligonucleotide as described in Example 19. Calmodulin-oligonucleotide is detected by addition of the quinoline indicator quin2 ad described by Tatsumi et al., 1997.

Example 24

Detection of a SH Labeled-Oligonucleotide Using Caspase Activity

Purified Caspase protease (Santa Cruz Biotechnologies, Santa Cruz, CA) is reacted with the 3'SH oligonucleotide to allow disulfide bond formation at the 3'end of the nucleic acid as described in Example 19. Unlabeled oligonucleotide is removed by size exclusion chromatography. Caspase activity is then detected by hydrolysis of the substrate Carboxybenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluromethyl coumarin (Enzyme System Products) at pH 7.2 as described by Stennick and Salvesen,

1997. Hydrolysis is monitored by the release of 7-amino-4-trifluromethyl coumarin as fluorescence emission at 505nm upon excitation at 400nm.

Example 25

Detection of a SH Labeled-Oligonucleotide Using Thermophilic Beta-NADH Oxidase

Purified NADH Oxidase from Thermus aquaticus (Athey, D. and McNeil, CJ, 1994) is reacted with the 3'SH oligonucleotide as described in Example 20. Excess NADH is added to the reaction and incubated at 60 degrees C. Oxidation is followed by concomitant two electron reduction of di-oxygen to hydrogen peroxide. Hydrogen peroxide is detected by oxidation at a platinum electrode as described by Athey, D. and McNeil, CJ, 1994).

Example 26

Detectionn of a SH Labeled-Oligonucleotide Using bð-Lactam Cleavage

Purified TEM-1 bð-lactamase is reacted with the 3 SH oligonucleotide to allow disulfide bond formation at the 3 end of the nucleic acid as described in Example 19. bð-lactamase activity is assayed using 1 mM nitrocefin (UniPath, chromophoric cephalosporin). Cleavage of the bð-lactam ring is monitored by the increase in absorbance at 482nm (eð482 =12,000 M-1 cm-1).

Example 27

Detection of a SH Labeled-Oligonucleotide Using the Copper Monoamine Oxidase Galactose Oxidase

Yeast copper amine Oxidase (Cai, D. and Klinmin, JP, 1994) is reacted with the 3'SH oligonucleotide to allow disulfide bond formation at the 3'end of the nucleic acid as described in Example 18. Amine oxidase activity is assayed using 5 mM methylamine as a substrate (Sigma). Oxygen consumption is monitored using an oxygen electrode (Yellow Springs Instrument).

Example 28

Detection of a SH Labeled-Oligonucleotide Using Reduction of NADH by Morphinone Reductase

Purified morphinone reductase (Craig et al., 1998) is reacted with the 3'SH oligonucleotide to allow disulfide bond formation at the 3'end of the nucleic acid as described in Example 19. Activity is measured by the reduction of excess NADH using the oxidizing substrate codeinone as described by Craig et al., 1998.

Example 29

Detection of a SH Labeled-Oligonucleotide Using Protein Kinase C Alpha

Protein Kinase C alpha (Life Technologies, Gaithersburg, MD) is reacted with the 3'SH oligonucleotide to allow disulfide bond formation at the 3'end of the nucleic acid as described in Example 19. Kinase activity is detected using a 13 amino acid peptide substrate with the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val. The contains the FRET-Quench pair described in Example 12 (Biosynthesis, Inc., Lewisville, TX). Cleavage of the labeled peptide allows fluorescence increase of the fluorescence donor similar to the method described in Example 24.

Example 30

Detection of a Biotin Array Oligonucleotide using a Selenium-Antibody Conjugate Optical Wave Guide

Biotin labeled array oligonucleotides are obtained from Biosynthesis, Inc., Lewisville, TX. Selenium colloloid and selenium-antibody conjugates are prepared and the hybridization and staining for the optical wave guides are performed as described by Stimpson et al., 1994.

Example 31

Detection of a SH Labeled-Oligonucleotide

Using Green Fluorescent Protein (GFP)

GFP is reacted with the 3'SH oligonucleotide to allow disulfide bond formation at the 3'end of the nucleic acid as described in Example 18. Fluorescent of GFP is detected directly.

Example 32

In this embodiment, a pre-formed circular vector is applied to single-stranded cDNA in order to identify and quantitate the RNA molecules in a population of RNA molecules obtained from normal and disease cells. A population of circular vectors is applied to gel pad arrays containing cDNA or RNA, columns and affinity chromatography using cDNA or RNA (see 5,714,320) or arrays of cDNA or RNA attached to a solid support (see, e.g., 5,503,980, Affymetrix and Synteni patents). The circular vectors include:

(1) A region of random DNA sequence (e.g., 5-50 bases, preferably 12 bases);

(2) A region containing a recognition sequence for a type IIS restriction enzyme that cleaves in the middle of the region of random DNA sequence (note: this region may be designed to form a hairpin or other structure as described in 5,714,320);

(3) Additional DNA sequence that is, ideally, not complementary to any of the target nucleic acid sequences (RNA or cDNA) such that the complete vector contains between 50-1500 bases.

Those circular vectors that recognize sequences in the target are separated from the population of circular vectors added to the target nucleic acids. Background hybridization can be minimized by including linear DNA that contains all of the vector sequence except for the region of random DNA. The isolated circular vectors are amplified using rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), the DNA is cleaving, e.g., using a restriction enzyme, and the resulting fragments are analyzed, e.g., interrogated on an indexing linker array (if dsDNA) see, e.g., 5,508,169 or a Cantor array (if ssDNA) see, e.g., 5,503,980. Preferably the analysis is performed in a 3 dimensional gel pad array, see, e.g., 5,552,270.

In another embodiment, circular vectors (as above) are used to identify the presence of mutations and SNPs by having a region of the circular DNA complementary to a mutation or SNP such that the circular DNA specifically binds to the mutation or SNP. Circular vectors complementary to a mutation or SNP will be isolated through application to a population of target DNA molecules (cDNA or RNA) e.g., bound to a solid support, a gel pad or a bead. The target DNA can be present as either an ordered array of distinct molecules, or as a non-ordered array of molecules on a solid support, a gel pad or a bead. The resulting vectors are amplified by rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), can be fragmented by restriction enzymes, and are analyzed, e.g., on an Indexing Linker (if dsDNA) see, e.g., 5,508,169 or a Cantor array (if ssDNA) see, e.g., 5,503,980.

Vectors can be separated into pools to prevent hybridization between the vectors (dsDNA probes should be avoided) and to maximize hybridization fidelity in any method described herein. The vector pools are applied to anchored target nucleic acid (genomic DNA, amplified DNA, cDNA or RNA) and those that hybridize to sequences in the target nucleic acid are isolated from the pool (conditions selected that maximize hybridization fidelity for each vector pool). The identity of the isolated vectors is determined by RCA, where the isolated oligo probes act as both a "positioning oligo" and an RCA primer (see 5,714,320). The DNA derived from rolling circle amplification (in the presence of a fluorescent nucleotides) is cleaved using a restriction enzyme, and the resulting fragments can be interrogated on an Indexing Linker array (if dsDNA) see, e.g., 5,508,169 or a Cantor array (if ssDNA) see. e.g., 5,503,980.

Example 33

A linear DNA vector probe is designed with two, random, e.g., 5mer, sequences in either end of the vector. There are 1024 possible 5mer sequences, so this would entail the synthesis of 1,048,576 linear vectors. The vectors will share one or a small number of common backbones, where each backbone can include a type IIS restriction site and a priming site for DNA synthesis. The vectors should be grouped such that the random 5mers in a given group of vectors can not be brought together by the common backbone sequence. The sequence of the target nucleic acid will then facilitate the circularization of a subset of the probe vectors, with each circularized probe vector representing a short contiguous, e.g., 10 basepair, stretch of target DNA. The DNA is amplified using RCA in the presence of fluorescent nucleotides. The single-stranded product of rolling circle amplification is rendered double-stranded by the annealing of un-circularized, complementary probe vector. The dsDNA RCA product is analyzed. It can be fragmented, e.g., using a typeIIS restriction enzyme such that the DNA is cleaved in the middle of the short region generated by the ligation reaction. The dsDNA fragments generated by the restriction

digest are analyzed, e.g., on an array of indexing linkers (see, e.g., 5,508,169). If the probe vector is labeled with a capture moiety, e.g., biotin group, then it is possible to render the dsDNA fragments generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture moiety reactive, e.g., substrate, e.g., strapavidin-labeled substrate, e.g., magnetic beads or solid support. The single-stranded DNA fragments can then be analyzed on a Cantor array. The DNA sequence of the target DNA is reconstructed using overlap analysis according to the procedure of Drmanac et al. (see, e.g., 5,525,464; 5,492,806; 5,202,231; 5,695,940).

Other embodiments are within the claims:

What is claimed is:

1. A method of analyzing a sample comprising:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally,

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and

(c) cleaving the oligonucleotide product to produce cleaved amplified product,

thereby analyzing a sample provided that: the oligonucleotide multimer is more sensitive to cleavage than is the circular template, the formation of said oligonucleotide product and its cleavage occur simultaneously or, at least one component, the TRCA primer, the sample, or the product is immobilized.

2. The method of claim 1, wherein the oligonucleotide multimer is more sensitive to cleavage than is the circular template.

3. The method of claim 1, wherein the formation of said oligonucleotide product and its cleavage occur simultaneously.

4. The method of claim 1, wherein at least one component, the TRCA primer, the sample, or the product is immobilized.

5. The method of claim 1, further comprising:

(3) analyzing said product from (2) b or c by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positionally distinguishable capture probe includes a unique region; and hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

6. The method of claim 1, wherein the circular template has one or more nucleotides or modified nucleotides which are resistant to cleavage.

7. The method of claim 1, wherein the circular template includes one or more of deoxy uracil, or a methylated or hemimethylated base.

8. The method of claim 1, wherein cleavage of the oligonucleotide multimer is effected by hybridization of a cleavage probe and said cleavage probe is chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

9. The method of claim 1, wherein the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

10. The method of claim 1, wherein the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

11. The method of claim 1, wherein the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template.

12. A method of analyzing a polynucleotide in a sample comprising:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2) (a) annealing an effective amount of sample sequence to a plurality of single-stranded circular templates, wherein each single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and optionally, (ii) at least one nucleotide effective to produce a cleavage site, and (iii) an identifier sequence which allows it to be distinguished from at least one other circular template of the plurality;

(b) combining the circular templates with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide product complementary to a circular oligonucleotide template, and

(c) cleaving the oligonucleotide product at the cleavage site to produce the cleaved amplified product, thereby analyzing the sample sequence.

13. The method of claim 12, further comprising:

(3) analyzing said product from (2) b or c by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positionally distinguishable capture probe includes a unique region complementing to an identifier sequence, and

hybridizing the product with the array of capture probes.

14. The method of claim 12, wherein the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

15. The method of claim 12, wherein the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

16. The method of claim 12, wherein the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template.

17. The method of claim 12, wherein the method detects a genetic event in the sample and the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

18. A method of analyzing a polynucleotide in a sample comprising:

(1) providing a sample which includes a sample polynucleotide sequence to be analyzed;

(2)(a) annealing an effective amount of sample sequence to a single-stranded circular template, wherein the single-stranded circular template comprises (i) at least one copy of a nucleotide sequence complementary to the sequence of the sample sequence and (ii) at least one nucleotide effective to produce a cleavage site;

(b) combining the circular template with an effective amount of a TRCA primer, at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template, wherein the oligonucleotide multimer comprises multiple copies (amplified) of the sample sequence; and optionally

(c) providing (i) an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal; or (ii) an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having having a second detecting moiety wherein the first and second detection moieties hybridize to the oligonucleotide

multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety; and

(3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

19. The method of claim 18, wherein the cleavage primer has one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product.

20. The method of claim 18, wherein the first detection oligonucleotide has a first detecting moiety and a second oligonucleotide has a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety.

21. The method of claim 18, wherein the signal increases upon cleavage at the cleavage site.

22. The method of claim 18, wherein the signal decreases upon cleavage at the cleavage site.

23. The method of claim 18, wherein the circular oligonucleic template (of any step) is prepared by a process comprising the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a single positioning oligonucleotide, e.g., a sample sequence, having a 5' nucleotide sequence complementary to a portion of the sequence comprising the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence comprising the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned so as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template

24. A rolling circle vector wherein the vector comprises:

(1) a first nucleic acid contacting region which is complementary to a first portion of a target nucleic acid;

a binding site for a type IIS restriction enzyme, wherein the cleavage site for the type IIS restriction enzyme is located within the first nucleic acid contacting region;

optionally,a primer target sequence, which can be used to provide for amplification of the vector when it circularized;

a second type IIS restriction enzyme binding site;

a second nucleic acid contacting region that is complimentary to a second portion of the target nucleic acid and which includes the cleavage site for the second type IIS restriction enzyme, wherein the first and second target portions are disposed such that upon hybridization of the vector to the target the ends of the vector can be joined; or

(2) a first nucleic acid contacting region which is complimentary to a first portion of a target nucleic acid (in preferred embodiments one or

more nucleotides of this region can vary in a population of vectors, for example one vector could have (t) at an interrogation position while another vector could have (a) at the interrogation position);

optionally a primer sequence which allows for rolling circle amplification of the vector;

optionally a spacer region;

a sequence tag region, which preferably includes four to ten nucleotide, which can allow for identification of the vector;

optionally a cleavage site for example a site for cleavage by a restriction enzyme;

a second nucleic acid contacting region which is complimentary to a second portion of the target nucleic acid, wherein the first and second target portions are disposed such that upon hybridization of the vector to the target the ends of the vector can be joined.

25. A method of providing a nucleotide fragment containing a target sequence compising:

providing a target nucleic acid sequence;

contacting the target nucleic acid sequence with the vector of claim 24 comprising a first nucleic acid contacting region which is complementary to a first portion of a target nucleic acid, and allowing the rolling circle amplification.

26. A plurality of the vectors of claim 24, wherein the plurality include the vector having a first nucleotide at the interrogation position and a vector having a second nucleotide at the interrogation position.

27. A method of analyzing a test nucleotide in a sample comprising:

(1) providing a sample which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2)(a) annealing an effective amount of sample polynucleotide sequence to a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally,

(b) combining the circular template with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and

(c) cleaving the product to produce cleaved amplified product,

wherein upon cleavage, the test nucleotide, or its complementary nucleotide, is sufficiently close to an end of the cleaved amplified product such that its presence can be detected by its effect on a reaction which involves the end nucleotide of the cleavage product.

28. The method of claim 27, wherein the reaction is selected from the group consisting of a hybridization reaction, a ligation reaction, a polymerization reaction, a restriction or other cleavage reaction.

29. The method of claim 27 wherein the test nucleotide, or its complementary nucleotide, is within 1, 2, 3, 4 or 5 base pairs from the end of the cleaved amplified product.

30. The method of claim 27, wherein the circular oligonucleic template is prepared by a process which includes the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a positioning oligonucleotide wherein the positioning oligonucleotide has a 5' nucleotide sequence complementary to a portion of the sequence which includes the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence which includes the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned such that they can be joined, and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template

31. The method of claim 27, wherein the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

32. The method of claim 27, wherein the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

33. The method of claim 27, further comprising analyzing a sample polynucleotide sequence includes sequencing at least one nucleotide of the polynucleotide sequence, sequencing by hybridization, or positional sequencing by hybridization.

34. The method of claim 27, wherein the method detects a genetic event in the sample and the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

35. The method of claim 27, further comprising:

analyzing said product from (2) b or c by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique region; and

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

36. A method of analyzing a test nucleotide in a sample comprising:

(1) providing a sample, which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2)(a) annealing an effective amount of sample polynucleotide sequence to an oligonucleotide, wherein the oligonucleotide includes preferably in a 5' to 3' orientation, a unique region which is complementary to a target sequence in the sample polynucleotide sequence, optionally a spacer region, a first pairing region, a connecting region which preferably is not homologous to the sample sequence, optionally a second spacer region, a second pairing region which is complementary to the first pairing region, and a second unique region complementary to a second adjacent sequence in the target polynucleotide; wherein hybridization of the unique regions to the sample promotes formation of a duplex between said first and second pairing regions, and wherein said duplex includes a cleavage site;

(b) cleaving the duplex to produce a cleaved product; and

(c) allowing the ends of the cleaved product to be joined, thereby forming a circular molecule,

wherein formation of the circular molecule is indicative of the presence of a sample sequence.

37. The method of claim 36, wherein the method further includes:

ligating the cleaved product to form a circle and using the circle to promote rolling circle amplification, e.g., to produce an oligonucleotide product.

38. The method of claim 36, wherein the method further includes:

providing a ligation template oligonucleotide which hybridizes to the cleaved product and thereby brings the ends of the cleaved product in proximity for a reaction, e.g., ligation.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18808

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 19/34; C12N 15/00; C07H 21/00, 21/02, 21/04
US CL :435/91.1, 91.2, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2, 320.1; 536/23.1, 24.32, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIZARDI et al. Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification. Nature Genetics. July 1998, Volume 19, No 3, pages 225-232. See the entire document.	1-38
A	WALTER, N.G. Modelling Viral Evolution *in Vitro* Using exo$^-$ Klenow Polymerase: Continuous Selection of Strand Displacement Amplified DNA that Binds an Oligonucleotide to Form a Triple-helix. Journal of Molecular Biology. 1995, Volume 254, pages 856-868. See entire document.	1-38
A, P	US 5,919,678 A (GRUSS et al.) 06 July 1999. See entire document.	1-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
08 NOVEMBER 1999	10 DEC 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
BRADLEY L. SISSON
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18808

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,714,320 A (KOOL) 03 February 1998. See entire document.	1-38
A, P	US 5,834,252 A (STEMMER et al.) 10 November 1998. See entire document.	1-38
A, E	US 5,942,391 A (ZHANG et al.) 24 August 1999. See entire document.	1-38
Y	US 5,648,245 A (FIRE et al.) 15 July 1997. See entire document.	1-38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18808

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST: US Patents; STN/CAS File Biosis, CA

Search Terms: rolling AND amplification; TRCA and primer?; e woodward k/au; e nallur g/au; e taylor s/au; vector and rolling amplification